Running Head:

Metabolic engineering of seed $\omega$-7 fatty acid accumulation

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Metabolic engineering of seeds can achieve levels of \( \omega-7 \) fatty acids comparable to the highest levels found in natural plant sources

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Abbreviations footnote

x:yΔz, fatty acid containing x carbons and y double bonds in position z counting from the carboxyl end; ACP, acyl carrier protein; CoA, coenzyme A; FA, fatty acid(s); FAS, fatty acid synthesis FAME, fatty acid methyl ester; KASII, β-ketoacyl-ACP synthase II; WT, wild type.

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Abstract:

Plant oils containing ω-7 fatty acids (FA) (palmitoleic 16:1 Δ⁹ and cis-vaccenic 18:1Δ¹¹) have potential as sustainable feedstocks for producing industrially important octene via metathesis chemistry. Engineering plants to produce seeds that accumulate high levels of any unusual FA has been an elusive goal. We have achieved high levels of ω-7 FA accumulation by systematic metabolic engineering of Arabidopsis thaliana. A plastidial 16:0-ACP desaturase has been engineered to convert 16:0 to 16:1Δ⁹ with specificity >100-fold that that of naturally-occurring paralogs such as that from Doxantha unguis-cati L. Expressing this engineered enzyme (Com25) in seeds increased ω-7 FA accumulation from <2% to 14%. Reducing competition for 16:0-ACP by downregulating the KASII 16:0 elongase further increased accumulation of ω-7 FA to 56%. The level of 16:0 exiting the plastid without desaturation also increased to 21%. Coexpression of a pair of fungal 16:0 desaturases in the cytosol reduced the 16:0 level to 11% and increased ω-7 FA to as much as 71%, equivalent to levels found in Doxantha seeds.
Introduction

There is increasing interest in the use of plant oils as renewable sources of industrial chemical feedstocks (Dyer et al., 2008; Carlsson, 2009). Recent developments in olefin metathesis have demonstrated that long chain monoene FA from vegetable oils can be efficiently split into the corresponding short chain alpha olefin and ω-alkenoic acids (Rybak et al., 2008; Meier, 2009). Thus ethenolytic metathesis of ω-7 FA such as palmitoleic or cis-vaccenic acids yields 1-octene and 9-decanolactone. 1-Octene is a high-demand feedstock with a global consumption of over half a million tonnes per year that is primarily used as a comonomer in the expanding production of linear low density polyethylene. It is mainly synthesized from petroleum-derived ethylene via oligomerization to yield a complex mixture of alpha olefins, or from coal-derived syngas (Systems, 2007).

A plant oil containing high (>70%) content of ω-7 FA would represent a new and sustainable feedstock for 1-octene production. Several natural plant oil sources of ω-7 FA have been reported, e.g., milkweed (Asclepias syriaca) accumulates ~25% ω-7 FA, (comprising ~10% 16:1 Δ9 and ~15% 18:1 Δ11) and cat’s claw vine (Doxantha unguis-cati) accumulates ~72% ω-7 FA, (comprising ~55% 16:1Δ9 and ~17% 18:1Δ11), but the low yields and poor agronomic properties of these plants preclude their commercial use for ω-7 FA production. Isolation of the Δ9-16:0-ACP desaturases genes responsible for the production of ω-7 FA from milkweed and Doxantha (Cahoon et al., 1997; Cahoon et al., 1998) presented an opportunity for their heterologous expression and ω-7 FA production in transgenic crops. However, heterologous expression of the milkweed desaturase in Arabidopsis failed to produce detectable ω-7 FA, and when the Doxantha desaturase was expressed in Brassica napus, it resulted in the accumulation of only ~9% ω-7 FA (Bondaruk et al., 2007). Thus, upon expression of either naturally occurring gene, ω-7 FA accumulation was far below that of the source plant suggesting additional metabolic modifications are required to optimize ω-7 FA accumulation in a host oilseed plant. Low levels of unusual FA accumulation upon the heterologous expression of variant desaturases is a generally observed phenomenon for monoene producing acyl-ACP desaturases (Suh et al., 2002) and efforts in this laboratory to express the Δ4-16:0 desaturases from coriander and ivy, and the Δ6-16:0 desaturase from Thunbergia alata in Arabidopsis
seed resulted in less than 3% of the target fatty acid accumulation in the best lines. Similarly, the accumulation of unusual fatty acids resulting from the expression of Fad2 integral membrane desaturase variants is much lower than the levels found in seeds of the native host (Napier, 2007). Together these observations constitute a significant barrier for biotechnological efforts to produce sustainable and renewable sources of oleochemical-derived feedstocks for products such as octene.

To overcome the problem of poor ω-7 FA accumulation, we performed a series of systematic metabolic engineering experiments to optimize the accumulation of ω-7 FA in transgenic plants. We chose to demonstrate proof of concept in the model oilseed plant Arabidopsis because its rapid generation time and facile transformation allowed us to rapidly quantify the effects of various combinations of transgenic approaches and mutant backgrounds. Based on the poor performance of naturally occurring Δ⁹-16:0-ACP desaturases, our first step was to identify a laboratory-derived variant desaturase with improved kinetics relative to its natural counterparts. We next systematically quantified the effects of various metabolic perturbations and then stacked the most promising traits into a single line. In doing so we demonstrated that coexpression of the laboratory-evolved plastidial desaturase, along with native extraplastidial 16:0Δ⁹-desaturases in a background in which the KASII elongase was strongly suppressed, resulted in increased accumulation of ω-7 FA from <2% in wild type (WT) to 71% in the best engineered line. This level of ω-7 FA is higher than that found in Asclepias and equivalent to that found in Doxantha seeds.
Results
In WT Arabidopsis, FA are synthesized de novo via the ACP pathway to a first branch point at the level of 16:0-ACP (Fig. 1). If acted on by FATB, the palmitoyl thioesterase, 16:0 free fatty acid (FA) is released from the plastid to the cytoplasm where it is esterified to coenzyme A (CoA), and subsequently transesterified onto phospholipids of the endomembrane system. Alternatively, β-ketoacyl-ACP synthase II (KASII) elongates the majority of 16:0-ACP to 18:0-ACP whereupon it is desaturated by Δ⁹-stearoyl-ACP desaturase to produce oleoyl-ACP. FATA, the oleoyl-ACP thioesterase, releases the oleic acid which exits the plastid and, like palmitate, becomes activated to the CoA-thioester and is transferred to phospholipids. In the ER, oleate can be elongated to 20:1Δ¹¹ via the action of fatty acid elongase (FAE) I, or become sequentially desaturated by the action of FAD2 and FAD3 to produce linoleic and linolenic acids respectively.

As Δ⁷-14:0-ACP desaturases are unknown, 16:0-ACP is the earliest metabolite in the FA synthesis pathway that can be intercepted and committed to ω-7 FA production by desaturation to 16:1Δ⁹-ACP. To achieve this we explored the feasibility of expressing a plastidial Δ⁹-16:0-ACP desaturase under the control of a seed-specific promoter (Fig. 1 reaction 1).

Choice of Δ⁹-16:0-ACP desaturase.
Several plants including Asclepias (Hopkins and Chisholm, 1961) and Doxantha (Chisholm and Hopkins, 1965) accumulate ω-7 FA in their seeds. Because the genes encoding these naturally occurring 16:0-ACP desaturases have been isolated (Cahoon et al., 1997; Cahoon et al., 1998), we first considered their use in efforts to engineer ω-7 FA accumulation. However, the measured in vitro activities of the desaturases from milkweed and Doxantha, like those of many variant desaturases, were considerably lower (milkweed 4% and Doxantha 11%) (Cahoon et al., 1997; Cahoon et al., 1998) than those reported for archetypal stearoyl-ACP desaturases acting on 16:0-ACP (Whittle and Shanklin, 2001). Test expression of these enzymes in Arabidopsis seed showed the Doxantha desaturase resulted in ~10% ω-7 FA, to similar levels as those previously reported upon its expression in Brassica (Bondaruk et al., 2007), while no product was detected upon expression of the milkweed enzyme in Arabidopsis.
We next considered the use of variants of the castor desaturase, including the previously reported 5.2 (Whittle and Shanklin, 2001), along with a battery of previously undescribed variants including Com25 that arose from enzyme evolution experiments designed to enhance the 16:0-desaturase activity of the castor Δ⁹-18:0-desaturase. Kinetic analysis was used to compare the various acyl-ACP desaturases; and Com25 was chosen because it exhibited the highest $k_{cat}/K_m$ (specificity factor) of 91 μM⁻¹ min⁻¹, i.e., equivalent to that of the castor WT desaturase for its natural 18:0-ACP substrate (Table 1).

**Expression of Com25 in WT Arabidopsis.** Expression of Com25 in transgenic seeds under the control of the seed-specific phaseolin promoter in WT Arabidopsis resulted in the accumulation of ~2% 16:1Δ⁹ and ~12% of its elongation product, 18:1Δ¹¹ yielding a total of ~14% ω-7 FA. See Fig. 2, panels A and B for a comparison of the gas chromatographic analysis of seed FA from WT Arabidopsis and WT expressing Com25.

**Expression of Com25 in Arabidopsis hosts containing increased levels of 16:0.**

As described above, β-ketoacyl-ACP synthase II (KASII) competes with Com25 for substrate and elongates 16:0-ACP to 18:0-ACP. We therefore sought lines with lowered KASII activity that would contain increased levels of 16:0 substrate (see Fig. 2. for representative GC traces of seed FA methyl esters)(James and Dooner, 1990). Despite many mutagenesis screens for altered fatty acid profiles, only one KASII mutant, fab1, has been reported (James and Dooner, 1990) that exhibits increased 16:0 levels in leaves and seeds. The seed 16:0 levels increase to ~21% of 16:0 from ~10% in WT (Fig. 2, C and Table 2). Biochemical evidence has confirmed the fab1 lesion is in KASII (Carlsson et al., 2002). Expression of Com25 in fab1 increased the accumulation of 16:1Δ⁹ and 18:1 Δ¹¹ to ~23% and ~16% respectively, yielding a total of ~39% ω-7 FA (Fig. 2, D and Table 2).

To simplify analysis we combined the fab1 and fae1 mutations because FAE1 is responsible for the extraplastidial elongation of 18:1Δ¹¹ to 20:1Δ¹³ (Fig. 1). The untransformed double mutant contains ~9% of ω-7 fatty acids, presumably reflecting increased desaturation of 16:0-ACP by the Δ⁹-18:0-ACP desaturase when presented with increased levels of 16:0-ACP substrate (Fig. 2, E and Table 2). Expression of Com25 in the fab1 fae1 background resulted in an increase of 16:1Δ⁹ and 18:1 Δ¹¹ to
~26% and ~23% respectively, yielding an increase of \( \omega-7 \) FA to ~50% (Fig. 2, F and Table 2). From the above results, increased accumulation of 16:1 in \( fab1 \) and \( fab1 \ fae1 \) mutant backgrounds correlates with increased 16:0, and so we sought additional lines in which 16:0 levels were elevated with respect to that of the \( fab1 \ fae1 \) double mutant. Two such lines were recently reported in which \( fab1 \) was suppressed, one by hairpin (HP)RNAi (Pidkowich et al., 2007) the other by a novel method of suppression termed hairpin-antisense (HPAS)RNAi (Nguyen and Shanklin, 2009). These lines contain strongly elevated seed 16:0 accumulation levels at 42% and 46% respectively (Fig 3.). Transformation with \( \text{Com25} \) yielded a further increase of ~5% in \( \omega-7 \) FA in both cases relative to the host background (Table 2). Thus, when \( \text{Com25} \) is expressed in host lines differing in 16:0 levels, the accumulation of \( \omega-7 \) FA is proportional to 16:0 levels up to ~30% with little difference observed in hosts accumulating 42 or 46% 16:0 (Fig. 3).

**Expression of extraplastidial \( \Delta^9-16:0 \) desaturases increases \( \omega-7 \) FA accumulation.**

The approach to increasing \( \omega-7 \) FA accumulation *via* the expression of a plastidial 16:0-ACP desaturase mimics the strategy employed by plants that naturally accumulate high levels of \( \omega-7 \) FA. As noted above, the use of a host Arabidopsis with elevated 16:0 correlates with the formation of \( \omega-7 \) FA. To test our hypothesis that the 16:0 had exited the plastid and was unavailable for \( \text{Com25} \) to desaturate we explored two approaches to reducing 16:0 accumulation in seed oil. One strategy was to increase levels of 16:0-ACP in the plastid by reducing the \( \text{FATB} \) thioesterase (Jones et al., 1995) activity responsible for the release of 16:0 from 16:0-ACP (Fig. 1). However, pilot experiments showed that suppression of \( \text{FatB} \) had only a minor effect on increasing \( \omega-7 \) FA accumulation and so we pursued an alternative approach of desaturating the 16:0 after export from the plastid. Upon exiting the plastid, free FA are esterified to Coenzyme A (CoA) by acyl Co-A synthases *en route* to their accumulation as triacylglycerols (Shockey et al., 2003). These cytoplasmic fatty acyl Co-As and phospholipid-linked FA constitute pools of substrates that could potentially be intercepted by extraplastidial desaturases. In preliminary experiments the expression of extraplastidial fungal desaturases from *Stagonospora nodorum* (Sn\( \Delta^9D \)) and *Aspergillus nidulans* (An\( \Delta^9D \)), either singly or in combination, were evaluated with respect to reducing 16:0 levels in Arabidopsis.
These desaturases are homologs of ole1, the well-characterized acyl-CoA Δ9-
desaturase from *Saccharomyces cerevisiae*. We observed that coexpression of the two
fungal desaturases under the control of the phaseolin promoter yielded promising
results in reducing 16:0 levels in WT Arabidopsis. We therefore tested the expression
of these desaturases along with *Com25* in a KASII HPAS-RNAi suppression line.
Expression of the extraplastidal fungal desaturases resulted in the conversion of
approximately half of the residual 16:0 to 16:1Δ⁹, resulting in a decrease of seed 16:0
from ~21% to ~11% (approximately the level seen in WT seeds) with a corresponding
increase in 16:1Δ⁹ from ~30% to ~43% (Table 2). The *fae1* mutant is almost entirely
devoid of 16:1Δ⁹ elongation activity so that levels of 18:1Δ¹¹ are unaffected in the
host *fab1 fae1 Com25* line when transformed with *SnΔ⁹D* and *AnΔ⁹D* (~25% and
23% respectively). Coexpression of plastidial and extraplastidial desaturases yielded
a mean accumulation of ~67% ω-7 FA, with individual lines showing greater than
71% ω-7 FA. Seeds of the highest ω-7 FA accumulating lines had equivalent oil
content to those of WT and germination, growth and development appeared
unaffected by the accumulation of ω-7 FA.

**Discussion**

It has been estimated that there may be upwards of 1,000 FA structures in nature
(Millar et al., 2000). Many of these FA are synthesized by an array of variants of
archetypal desaturases (Shanklin and Cahoon, 1998). The isolation of many of these
desaturases and desaturase-like enzymes led to the expectation that their heterologous
expression would lead to the production of the corresponding unusual FA at high
levels. After more than a decade of effort, the production of commercial levels of
unusual FA based on the expression of variant desaturase enzymes has yet to be
reported (Napier, 2007). A contributing factor may be that unusual FA producing
acyl-ACP desaturase enzymes have *in vitro* turnover numbers one or two orders of
magnitude below those of the archetypal desaturases from which they evolved. This
may reflect the fact that natural evolution proceeds *via* random mutagenesis, in which
achieving the combinations of specific changes at specific amino acid locations
necessary to effect changes in functionality is commonly accompanied by a large
number of changes distributed throughout the gene, some of which degrade the
enzyme’s kinetic properties. For instance, the *Doxantha* desaturase, the more active
of the two naturally-occurring desaturases investigated herein, (Table 1) has a $k_{cat}$ for 16:0 that is 135-fold lower than the ~1 sec$^{-1}$ of the castor WT desaturase with 18:0 substrate and 35-fold lower than the castor desaturase variant Com25 with 16:0 substrate. Others have suggested the poor in vitro performance of variant acyl-ACP desaturases may reflect a requirement for specific ferredoxins or ACPs (Suh et al., 1999; Schultz et al., 2000). However, despite work in our and other labs, to date there are no reports that coexpression of such factors has enhanced unusual FA accumulation.

Com25 exhibits a specificity factor for 16:0 equivalent to that of the castor WT desaturase for its natural 18:0 substrate. Thus a key factor in the successful production of high levels of $\omega$-7 FA in this study is the use of Com25 with its improved $K_m$ for 16:0-ACP allowing it to compete more effectively with FatB (the 16:0-ACP thioesterase) and KASII (the 16:0-ACP elongase), for substrate (see Fig. 1). That $\omega$-7 FA increases proportionately with 16:0 levels up to ~30% suggests substrate is limiting in this range above which Com25, ferredoxin, reductant or molecular oxygen may become limiting.

The strategy of expressing a 16:0-acyl-ACP desaturase to accumulate $\omega$-7 FA was designed to mimic the strategy employed by naturally-occurring $\omega$-7 FA accumulating plants. The suppression of the KASII elongase activity resulted in increased release of 16:0 from ACP by FATB (Salas and Ohlrogge, 2002). FA exiting the plastid traverse the cytosolic acyl-CoA pool en route to storage as triacylglycerols, presenting an additional opportunity to convert 16:0 to 16:1 in the cytoplasm. Coexpression of fungal 16:0-CoA desaturases facilitated the conversion of ~50% of the residual 16:0 into 16:1$\Delta^9$ product.

Here we demonstrate proof of concept in Arabidopsis seeds for a strategy to accumulate desired $\omega$-7 monoene products to industrially relevant levels. Different strategies will likely be necessary to optimize the accumulation of oxygenated FA such as ricinoleic or vernolic acid (Lee et al., 1998) for which downstream enzymes such as acyltransferases with improved specificity for the unusual FA appear to be important for improving the accumulation of the unusual FA (Burgal et al., 2008). In another example involving the production of laurate in canola, the coexpression of a coconut lysophosphatidic acid acyltransferase with the California bay medium chain thioesterase enhanced laurate accumulation from 50% to 60% in the best example of
unusual FA production to date (Knutzon et al., 1999). It is possible that the levels of ω-7FA reported herein could be further optimized by expression of appropriate acyltransferases or by fine tuning the expression of thioesterases.

**Conclusion**

The optimization of any unusual FA will likely require the stacking of multiple traits (Napier, 2007). Thus our approach of 1) identifying and expressing a natural or engineered desaturase with favorable kinetic parameters, 2) quantifying incremental improvements resulting from additional transgene coexpression, 3) modulation of endogenous activities, followed by 4) trait stacking, is a general strategy that will likely result in the successful optimization of a variety of unusual FA.

Specifically, to optimize the accumulation of ω-7 FA we: 1) Introduced an engineered enzyme optimized for its specificity factor (kcat/Km) capable of competing with endogenous enzymes for substrate *in vivo*. 2) Optimized the level of substrate by manipulating the levels of the competing enzyme KASII. 3) Increased product yield by expressing additional and compartment-specific 16:0-CoA desaturases.

In summary, we have exemplified a strategy for metabolically engineering the sustainable production of an ω-7 FA feedstock for the production of a high-demand product octene, in higher plants.

**Material and Methods**

**Arabidopsis growth and transformation.**

*Arabidopsis* plants were grown in soil under continuous exposure to 300 microeinsteins of light (1 microeinstein = 1 mol of light) in E7/2 controlled environment growth chambers (Conviron). The plants were transformed according to Clough and Bent’s method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain GV3101. We identified individual T1 seeds carrying the transgenes by the fluorescence emitted (Stuitje et al., 2003) upon illumination with green light from an X5 LED flashlight (Inova) in conjunction with a 25A red camera filter (Pidkowich et al., 2007). A WILD M3Z dissection microscope equipped with an Olympus U-LH100HG illumination system was used to discriminate between seeds carrying Zs-Green and Ds-Red markers with the use of filters FITC 535 and FITC 515.
respectively. Seed-specific expression was achieved by placing constructs under the control of the phaseolin seed storage protein promoter or the LTP170 promoter (Slightom et al., 1983; van der Geest and Hall, 1997).
Source of Com25.

Com25 is a variant of the Ricinus communis Δ⁹-18:0-ACP desaturase that arose from a program of combinatorial saturation mutagenesis/selection designed to identify variants with improved activity towards acyl chains of <18C in lengths (Whittle and Shanklin, 2001). Com25 differs from the parental castor desaturase at the following 5 amino acid positions: M114S, T117R, L118C, P179L, G188T (numbered according to the mature castor desaturase PDB entry 1AFR).

Plasmid constructs

Phas:Com25

The entire open reading frame of the castor variant Com25, engineered to contain its authentic transit peptide and flanked by 5’ PacI and 3’ XhoI restriction sites was cloned into the corresponding sites of plasmid pDs-Red-Phas (Pidkowich et al., 2007) (with Ds-Red marker) to created Phas:Com25 (Fig. 5).

Phas:Fab1-HPAS

Construction of the Phas:Fab1-HPAS construct was described previously (Nguyen and Shanklin, 2009).

Phas:FatB-HPAS

This construct was created in two steps, first the construction of Phas:FatB-HP, and afterwards the insertion of an antisense portion of the FatB gene to replace part of the Fad2 intron separating the sense and antisense portions of the FatB gene comprising the hairpin. To achieve this, 150 bp of the Arabidopsis FatB 3’UTR was amplified from genomic DNA in both sense (using primers FatB-hps-5’PstI GGGCTGCAGAAACAAGTTCGGCCACCAACCC and FatB-hps-3’XhoI CCCCTCGAGACATCAGAATTCGTAATGAT) and antisense (using primers FatB-hpa-5’NheI GGGGCTAGCAAGTTTCGGCCACCAACCC and FatB-hpa-3’PacI CCCTTAATTAAACATCAGAATTCGTAATGAT) orientations. These fragments were restricted with PstI/XhoI and NheI/PacI and used to replace the 5’UTR sense and antisense portions of Fab1 in pGEM-T-Easy-HTM3 (Pidkowich et al., 2007) at their equivalent sites, to create the intermediate plasmid pGEM-T-Easy-HTM4. To create a 300 bp antisense portion of the FatB coding
region, a fragment was amplified with primers FatB-Exon-5'Sp-Bam
(CCACTAGTGGATCCACCTCTCTCTACGTCGTCATT) and FatB-Exon-3'Bg-Sal
(GGAGATCTGTCGACGTTATATAGTAGCAAGAAG), and the fragment,
restricted with BamHI and SalI, was used to replace part of the Fad2-intron after
restriction with BglII and SpeI to create pGEM-T-Easy-HTM5.
The assembled HPAS fragment was excised with the use of PacI and XhoI and cloned
into the equivalent sites of pZs-Green-Phas:Com25 (plasmid pDs-Red-Phas:Com25,
described above, in which the fluorescence marker pCVMV:Ds-Red had been
replaced by a green fluorescent protein maker pCVMV:Zs-Green (Clonetech)) to
create plasmid Phas:FatB-HPAS (Fig. 5).

**Phas:AnΔ9D, Phas:SnΔ9D**

Two fungal acyl-CoA Δ9 desaturases were combined in plasmid pDAB7318 with
both genes being driven by the seed-specific Phas promoter from *Phaseolus vulgaris*.
The first gene in the construct was an acyl-CoA Δ9-desaturase from *Aspergillus
nidulans* that was redesigned and synthesized for optimal expression in plants and
fused to the 3’ untranslated region and 3’ MAR from the *Phaseolus vulgaris*
phaseolin gene. The second desaturase gene in this construct was an acyl-CoA Δ9-
desaturase from *Stagonospora nodorum* that was also redesigned and synthesized for
plant expression and fused to the *Agrobacterium tumefaciens* ORF23 3’ untranslated
region (Fig. 5). This desaturase was identified by homology searches of the *S.
nodorum* genome sequence released by the *Stagonospora nodorum* Sequencing
Project, Broad Institute of Harvard and MIT (http://www.broad.mit.edu). It was
shown to have a preference for desaturation of palmitate by complementation of the
*ole1* mutant of *Saccharomyces cerevisiae* (Stukey et al., 1990).

**Phas:Fab1-HPAS, Phas:Com25**

To simplify gene stacking experiments, we constructed plasmid Phas:Fab1/HPAS,
Phas:Com25 to combine Com25 expression with KASII suppression. To achieve this
the EcoRV-EcoRV fragment containing the Phaseolin promoter driving Com25 along
with the Phaseolin terminator was isolated from Phas:Com25 and cloned into the
intermediate vector pBL to create pBL-Phas:Com25-PhasTer. This Com25
expression cassette was excised using flanking EcoRI-EcoRI restriction sites and
cloned into the corresponding site within Phas:Fab1-HPAS to create Phas:Fab1-HPAS-Phas-Com25 (Fig. 5)

**Fatty-acid analysis**

To analyze the fatty acids of single seeds, we prepared fatty-acid methyl esters (FAMEs) by incubating the seeds with 0.2M trimethylsulfonium hydroxide in methanol (Butte et al., 1982). To similarly analyze bulk seeds, FAMEs were prepared by incubation them in 0.5 ml BCl₃ for 1h at 80°C, extracting them with 1 ml of hexane and then drying under N₂. FAMEs were analyzed either with an HP6890 gas chromatograph-flame ionization detector (Agilent Technologies) or an HP5890 gas chromatograph-mass spectrometer (Hewlett–Packard) fitted with 60-m x 250-µm SP-2340 capillary columns (Supelco). The oven temperature was raised during the analyses 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). We determined the double-bond positions of mono-unsaturated FAMEs by dimethyl disulfide derivatization (Yamamoto et al., 1991).

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GC-MS as their dimethyl disulfide adducts. Chem. and Phys. Lipids 60: 39-50
Figure Legends

Figure 1. Schematic of fatty acid synthesis and modification in the plastid and endoplasmic reticulum of Arabidopsis. Reactions mediated by 16:0 desaturases are indicated 1, Δ9-16:0-ACP desaturase, 2, extraplastidial Δ9-16:0-desaturase. ω-7 FA of interest in this study, i.e., 16:1 Δ9 and 18:1 Δ11 are boxed.

Figure 2. Representative gas chromatographic separation of FAMEs upon expression of Com25 in various backgrounds of Arabidopsis. Panels A and B, WT; C and D, fab1; E and F, fab1 fad1. Panels A, C and E, untransformed; B, D and F, transformed with Phas:Com25. FAME peaks are indicated: 16:0 (1), 16:1Δ9 (2), 16:2 (3), 18:0 (4), 18:1 Δ9 (5), 18:1 Δ11 (6), 18:2 (7), 20:0 (8), 20:1 Δ11 (9), 18:3+20:1 Δ13 (10), 22:1 (11)

Figure 3. Relationship between 16:0 in host seeds versus ω-7 FA accumulation (as mol percent).

Figure 4. Representative chromatograms of FAMES from the best fab1 fad1, Phas:Com25, Fab1-HPAS, AnΔ9DS, SnΔ9DS transformant line (Panel A); and Doxantha seed (Panel B). Peak designations are as described in Fig. 2.

Figure 5. Schematic arrangement of DNA elements in constructs described in this manuscript. Numbers indicate size in bp.
Table I
Comparison of the kinetic parameters of the castor desaturase and its variants with that of Doxantha.

| Enzyme | Substrate | $k_{cat}^{a}$ | $K_m$ | specificity factor
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<td></td>
<td></td>
<td>min$^{-1}$b</td>
<td>μM</td>
<td>μM$^{-1}$.min$^{-1}$</td>
</tr>
<tr>
<td>Com25</td>
<td>16:0-ACP</td>
<td>11.1 (0.6)</td>
<td>0.12 (0.03)</td>
<td>91</td>
</tr>
<tr>
<td>5.2$^c$</td>
<td>16:0-ACP</td>
<td>25.3 (1.1)</td>
<td>0.55 (.06)</td>
<td>46</td>
</tr>
<tr>
<td>WT$^c$</td>
<td>16:0-ACP</td>
<td>2.8 (0.1)</td>
<td>5.0 (0.5)</td>
<td>0.56</td>
</tr>
<tr>
<td>WT$^c$</td>
<td>18:0-ACP</td>
<td>42.3 (1.6)</td>
<td>0.46 (0.05)</td>
<td>92</td>
</tr>
<tr>
<td>Doxantha</td>
<td>16:0-ACP</td>
<td>0.31 (0.03)</td>
<td>0.43 (0.12)</td>
<td>0.72</td>
</tr>
</tbody>
</table>

$^{a}$ $k_{cat}$ is reported per diiron site
$^{b}$ Mean with S.E. in parentheses, n=10.
$^{c}$ data from ref. 14
Table II.
Comparison of the fatty acid composition of various lines of transgenic Arabidopsis seeds with those of *Doxantha*.

*a* Tot ω-7 fatty acids, the mole percent of 16:1Δ⁹ + 18:1 Δ¹¹ fatty acid species; double bond positions validated by their co-elution with authentic standards upon gas chromatographic separation and by mass spectrometry of their pyrrolidide derivatives.

<table>
<thead>
<tr>
<th>genotype</th>
<th>Fatty Acid Species</th>
<th>Tot ω-7 b</th>
<th>Δ ω-7 b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>16:1Δ9</td>
<td>16:2</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4±0.4 c</td>
<td>0.1±0.06</td>
<td>0</td>
</tr>
<tr>
<td>fab1</td>
<td>20.8±1.6</td>
<td>1.5±0.5</td>
<td>0</td>
</tr>
<tr>
<td>fab1, fael</td>
<td>26.8±1.9</td>
<td>1.9±0.3</td>
<td>0</td>
</tr>
<tr>
<td>WT, com25</td>
<td>9.2±1.5</td>
<td>1.6±0.4</td>
<td>0</td>
</tr>
<tr>
<td>fab1, com25</td>
<td>18.6±2.1</td>
<td>23.5±3.7</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>fab1, fael, com25</td>
<td>22±2.6</td>
<td>26.2±2.9</td>
<td>2.0±0.4</td>
</tr>
<tr>
<td>fab1, fael, com25, Fab1-HPAS</td>
<td>20.7±2.1</td>
<td>30.3±1.6</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>fab1, fael, AnD9D,SnD9D</td>
<td>12.7±2.1</td>
<td>17.9±1.8</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>fab1, fael, Fab1-HPAS com25, AnD9D,SnD9D</td>
<td>11.2±1.3</td>
<td>43.4±3.3</td>
<td>0.7±0.5</td>
</tr>
<tr>
<td>Doxantha</td>
<td>18.0±0.5</td>
<td>54.6±1.7</td>
<td>2.4±0.5</td>
</tr>
</tbody>
</table>

Δ ω-7, difference between the parental line and the line containing the introduced element as indicated by underlining in the genotype column.

Values represent mean ± SE, n=10 (or greater) of homozygous lines, verified for at least two generations.
**Figure 1.** Schematic of fatty acid synthesis and modification in the plastid and endoplasmic reticulum of Arabidopsis. Reactions mediated by 16:0 desaturases are indicated: 1, Δ9-16:0-ACP desaturase, 2, extraplastidial Δ9-16:0 desaturase. W.7 FA of interest in this study, i.e., 16:1 Δ9 and 18:1 Δ11 are boxed.
Figure 2. Representative gas chromatographic separation of FAMEs upon expression of Com25 in various backgrounds of Arabidopsis. Panels A and B, WT; C and D, fab1; E and F, fab1 fae1. Panels A, C and E, untransformed; B, D and F, transformed with Phas:Com25. FAME peaks are indicated: 16:0 (1), 16:1Δ^9 (2), 16:2 (3), 18:0 (4), 18:1Δ^9 (5), 18:1Δ^11 (6), 18:2 (7), 20:0 (8), 20:1Δ^11 (9), 18:3Δ^6,18:4 (10), 22:1Δ^11 (11).
Figure 3. Relationship between 16:0 in host seeds versus ω-7 PFA accumulation (as mol percent).
Figure 4. Representative chromatograms of FAMES from the best fab1 fae1, Phas:Com25, Fab1-HPAS, AnD9DS, SnD9DS transformant line (Panel A); and Doxantha seed (Panel B). Peak designations are as described in Fig. 2.
Figure 5. Schematic arrangement of DNA elements in constructs described in this manuscript, numbers represent b.p.