Metabolic Engineering of Seeds Can Achieve Levels of ω-7 Fatty Acids Comparable with the Highest Levels Found in Natural Plant Sources[1][OA]

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Plant oils containing ω-7 fatty acids (FAs; palmitoleic 16:1Δ^9 and cis-vaccenic 18:1Δ^{11}) 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 achieved high levels of ω-7 FA accumulation by systematic metabolic engineering of Arabidopsis (*Arabidopsis thaliana*). A plastidial 16:0-ACP desaturase has been engineered to convert 16:0 to 16:1Δ^9 with specificity >100-fold than that of naturally occurring paralogs, such as that from cat's claw vine (*Doxantha unguis-cati*). Expressing this engineered enzyme (Com25) in seeds increased ω-7 FA accumulation from <2% to 14%. Reducing competition for 16:0-ACP by down-regulating the β-ketoacyl-ACP synthase II 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.

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 α-olefin and ω-alkenoic acids (Rybak et al., 2008; Meier, 2009). Thus, ethenolytic metathesis of ω-7 fatty acids (FAs), such as palmitoleic or cis-vaccenic acids, yields 1-octene and 9-decenoate. 1-Octene is a high-demand feedstock with a global consumption of over half a million tons 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 α-olefins or from coal-derived syngas (Anonymous, 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 approximately 25% ω-7 FA (comprising approximately 10% 16:1Δ^9 and approximately 15% 18:1Δ^{11}) and cat's claw vine (*Doxantha unguis-cati*) accumulates approximately 72% ω-7 FA (comprising approximately 55% 16:1Δ^9 and approximately 17% 18:1Δ^{11}), but the low yields and poor agronomic properties of these plants preclude their commercial use for ω-7 FA production. Isolation of the Δ^9-16:0-ACP desaturases genes responsible for the production of ω-7 FA from milkweed and *Doxantha* (Cahoon et al., 1997, 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 (*Arabidopsis thaliana*) failed to produce detectable ω-7 FA, and when the *Doxantha* desaturase was expressed in *Brassica napus*, it resulted in the accumulation of only approximately 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 that 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 Δ^4-16:0 desaturases from coriander (*Cor-*

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iandrum sativum) and ivy (*Hedra helix*) and the Δ^6-16:0 desaturase from *Thunbergia alata* in Arabidopsis seed resulted in <3% of the target FA accumulation in the best lines. Similarly, the accumulation of unusual FAs 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 Δ^9-16:0-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 16:0Δ^9-desaturases in a background in which the β-ketoacyl-ACP synthase II (KASII) elongase was strongly suppressed, resulted in increased accumulation of ω-7 FA from <2% in the wild type 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 wild-type Arabidopsis, FAs 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 fatty acid thioesterase B (FATB), the palmitoyl thioesterase, 16:0 free FA is released from the plastid to the cytoplasm, where it is esterified to CoA and subsequently transesterified onto phospholipids of the endomembrane system. Alternatively, KASII elongates the majority of 16:0-ACP to 18:0-ACP, whereupon it is desaturated by Δ^9-stearoyl-ACP desaturase to produce oleoyl-ACP. FATA, the oleoyl-ACP thioesterase, releases the oleic acid that exits the plastid and, like palmitate, becomes activated to the CoA-thioester and is transferred to phospholipids. In the endoplasmic reticulum, oleate can be elongated to 20:1Δ^{11} via the action of fatty acid elongase 1 (FAE1) or becomes sequentially desaturated by the action of FAD2 and FAD3 to produce linoleic and linolenic acids, respectively.

As Δ^7-14:0-ACP desaturases are unknown,16:0-ACP is the earliest metabolite in the FA synthesis pathway

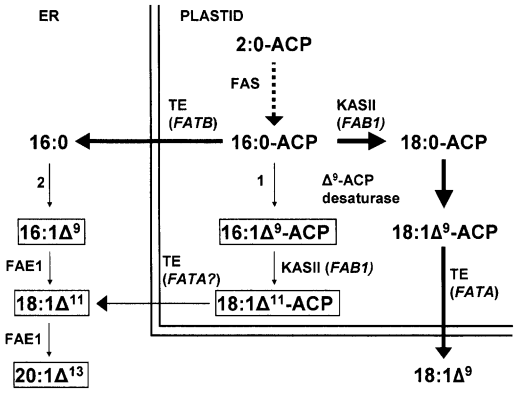

Figure 1. Schematic of FA synthesis and modification in the plastid and endoplasmic reticulum of Arabidopsis. Reactions mediated by 16:0 desaturases are indicated: 1, Δ^9-16:0-ACP desaturase; 2, extraplastidial $\Delta^9$16:0-desaturase. ω-7 FAs of interest in this study, i.e. 16:1Δ^9 and 18:1Δ^{11}, are boxed.

that can be intercepted and committed to ω-7 FA production by desaturation to 16:1Δ^9-ACP. To achieve this, we explored the feasibility of expressing a plastidial Δ^9-16:0-ACP-desaturase under the control of a seed-specific promoter (Fig. 1, reaction 1).

Choice of Δ^9-16:0-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, 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, 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 approximately 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 Δ^9-18:0-desaturase. Kinetic analysis was used to compare the various acyl-ACP desaturases; Com25 was chosen because it exhibited the highest k_{cat}/K_m (specificity factor) of 91 μM^{-1} min^{-1}, i.e. equivalent to

Table I. *Comparison of the kinetic parameters of the castor desaturase and its variants with those of Doxantha*

Enzyme	Substrate	k_{cat}[a]	K_m	Specificity Factor k_{cat}/K_m
		min^{-1}[b]	μM	$\mu M^{-1}\ min^{-1}$
Com25	16:0-ACP	11.10 (0.60)	0.12 (0.03)	91.00
5.2[c]	16:0-ACP	25.30 (1.10)	0.55 (0.06)	46.00
Wild type[c]	16:0-ACP	2.80 (0.10)	5.00 (0.50)	0.56
Wild type[c]	18:0-ACP	42.30 (1.60)	0.46 (0.05)	92.00
Doxantha	16:0-ACP	0.31 (0.03)	0.43 (0.12)	0.72

[a]k_{cat} is reported per di-iron site. [b]Mean with SE in parentheses (n = 10). [c]Data from Whittle and Shanklin (2001).

that of the castor wild-type desaturase for its natural 18:0-ACP substrate (Table I).

Expression of *Com25* in Wild-Type Arabidopsis

Expression of Com25 in transgenic seeds under the control of the seed-specific phaseolin promoter in wild-type Arabidopsis resulted in the accumulation of approximately 2% 16:1Δ^9 and approximately 12% of its elongation product, 18:1Δ^{11}, yielding a total of approximately 14% ω-7 FA. For a comparison of the gas chromatographic analysis of seed FA from wild-type Arabidopsis and the wild type expressing Com25, see Figure 2, A and B.

Expression of *Com25* in Arabidopsis Hosts Containing Increased Levels of 16:0

As described above, KASII competes with Com25 for substrate and elongates 16:0-ACP to 18:0-ACP. Therefore, we 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 FA 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 approximately 21% from approximately 10% in the wild type (Fig. 2C; Table II). Biochemical evidence has confirmed that the *fab1* lesion is in KASII (Carlsson et al., 2002). Expression of *Com25* in *fab1* increased the accumulation of 16:1Δ^9 and 18:1Δ^{11} to approximately 23% and approximately 16% respectively, yielding a total of approximately 39% ω-7 FA (Fig. 2D; Table II).

To simplify analysis, we combined the *fab1* and *fae1* mutations because FAE1 is responsible for the extraplastidial elongation of 18:1Δ^{11} to 20:1Δ^{13} (Fig. 1). The untransformed double mutant contains approximately 9% of ω-7 FAs, presumably reflecting increased desaturation of 16:0-ACP by the Δ^9-18:0-ACP desaturase when presented with increased levels of 16:0-ACP substrate (Fig. 2E; Table II). Expression of *Com25* in the *fab1 fae1* background resulted in an increase of 16:1Δ^9 and 18:1Δ^{11} to approximately 26% and approximately 23% respectively, yielding an increase of ω-7 FA to approximately 50% (Fig. 2F; Table II).

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

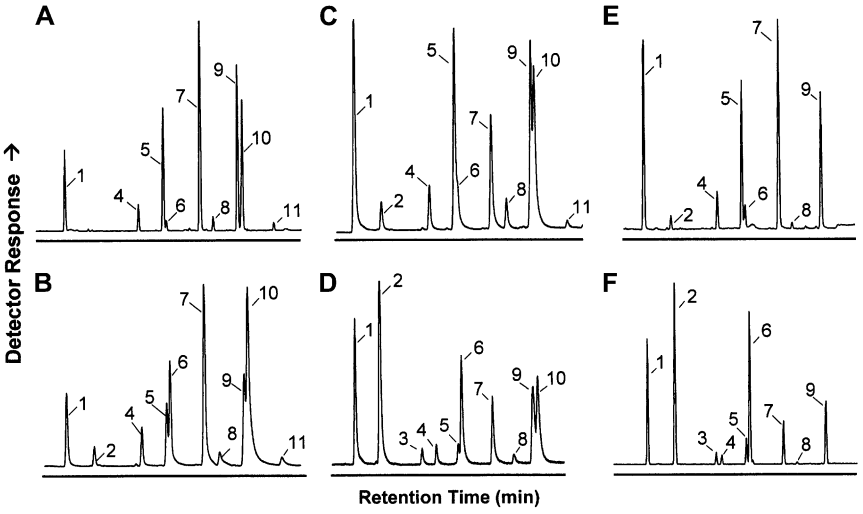

Figure 2. Representative gas chromatographic separation of FAMEs upon expression of *Com25* in various backgrounds of Arabidopsis. A and B, the wild type; C and D, *fab1*; E and F, *fab1 fae1*. A, C, and E, Untransformed; B, D, and F, transformed with Phas:*Com25*. FAME peaks are indicated: 16:0 (1), 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), 18:3+20:1Δ^{13} (10), and 22:1 (11).

Table II. *Comparison of the FA composition of various lines of transgenic Arabidopsis seeds with those of Doxantha*

Genotype	FA Species										Total ω-7[a]	Δ ω-7[b]
	16:0	16:1Δ9	16:2	18:0	18:1Δ9	18:1Δ11	18:2	18:3	20:0	20:1		
Wild type	10.4 (0.4)[c]	0.1 (0.06)	0	3.7 (0.4)	14.5 (2.2)	1.7 (0.2)	26.6 (0.9)	20.2 (1.1)	2.1 (0.3)	20.7 (0.6)	1.8 (0.2)	
fab1	20.8 (1.6)	1.5 (0.5)	0	3.7 (0.4)	18.4 (1.3)	2.8 (0.9)	14.8 (2.7)	19.1 (1.3)	3.1 (0.8)	15.8 (1.0)	4.3 (1.3)	2
fab1, *fae1*	26.8 (1.9)	1.9 (0.3)	0	3.6 (0.5)	15.8 (1.5)	6.8 (0.8)	25.3 (2.5)	19.7 (1.5)	0.2 (0.1)	0	8.7 (1.1)	4
Wild type, Com25	9.2 (1.5)	1.6 (0.4)	0	3.7 (0.3)	5.7 (1.6)	12.8 (1.2)	26.6 (1.6)	29.6 (2.2)	2.1 (0.4)	8.7 (2.1)	14.4 (1.5)	12
fab1, Com25	18.6 (2.1)	23.5 (3.7)	1.6 (0.5)	1.9 (0.3)	2.9 (2.2)	15.6 (3.3)	9.6 (1.7)	17.1 (1.8)	1.0 (0.4)	8.2 (3.4)	39.1 (1.9)	35
fab1, *fae1*, Com25	22 (2.6)	26.2 (2.9)	2.0 (0.4)	1.8 (0.4)	3.7 (1.1)	23.4 (2.3)	8.6 (0.7)	12.4 (1.8)	0	0	49.6 (1.1)	41
fab1, *fae1*, Com25, *Fab1-HPAS*	20.7 (2.1)	30.3 (1.6)	2.2 (0.2)	0.9 (0.6)	4.7 (1.4)	24.5 (1.8)	6.6 (0.9)	10 (1.6)	0	0	54.8 (1.5)	5
fab1, *fae1*, *AnD9D,SnD9D*	12.7 (2.1)	17.9 (1.8)	0.8 (0.1)	0.2 (0.1)	17.7 (0.9)	5.8 (0.7)	24.1 (1.2)	20.8 (0.9)	0	0	23.7 (1.9)	15
fab1, *fae1*, *Fab1*-HPAS Com25, *AnD9D,SnD9D*	11.2 (1.3)	43.4 (3.3)	0.7 (0.5)	0.9 (0.2)	4.6 (1.5)	23.2 (1.1)	8.4 (1.2)	7.6 (1.6)	0	0	66.6 (3.9)	12
Doxantha	18.0 (0.5)	54.6 (1.7)	2.4 (0.5)	2.0 (0.1)	1.8 (0.2)	17.3 (1.5)	3.9 (0.7)	0	0	0	71.9 (1.3)	

[a]Total ω-7 FAs, the mol % of 16:1Δ^9 + 18:1Δ^{11} FA species; double bond positions validated by their coelution with authentic standards upon gas chromatographic separation and by mass spectrometry of their pyrrolidide derivatives. [b]Δ ω-7, difference between the parental line and the line containing the introduced element as indicated by underlining in the genotype column. [c]Values represent mean (±SE), n = 10 (or greater).

were recently reported in which *fab1* was suppressed, one by hairpin RNA interference (RNAi; Pidkowich et al., 2007) and 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 *Com25* yielded a further increase of approximately 5% in ω-7 FA in both cases relative to the host background (Table II). Thus, when Com25 is expressed in host lines differing in 16:0 levels, the accumulation of ω-7 FA is proportional to 16:0 levels up to approximately 30% with little difference observed in hosts accumulating 42 or 46% 16:0 (Fig. 3).

Expression of Extraplastidial Δ^9-16:0 Desaturases Increases ω-7 FA Accumulation

The approach to increasing ω-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 ω-7 FA. As noted above, the use of a host Arabidopsis with elevated 16:0 correlates with the formation of ω-7 FA. To test our hypothesis that the 16:0 had exited the plastid and was unavailable for 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 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 FatB had only a minor effect on increasing ω-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 FAs are esterified to CoA by acyl-CoA synthases en route to their accumulation as triacylglycerols (Shockey et al., 2003). These cytoplasmic fatty acyl-CoAs 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*Δ9D) and *Aspergillus nidulans* (*An*Δ9D), either singly or in combination, was 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 wild-type Arabidopsis. We therefore tested the expression of these desaturases along with *Com25* in a KASII HPAS-RNAi suppression line. Expression of the extraplastidial fungal desaturases resulted in the conversion of approximately half of the residual 16:0 to 16:1Δ^9,

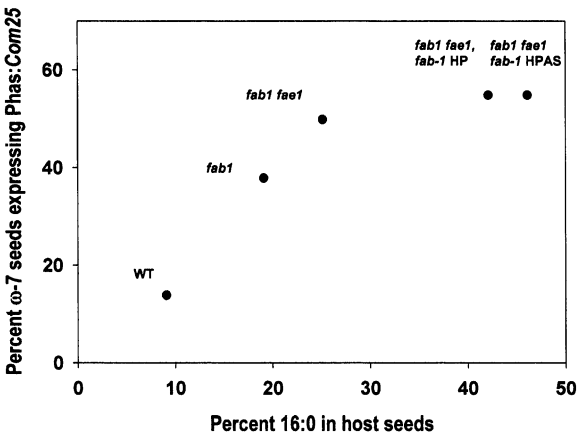

Figure 3. Relationship between 16:0 in host seeds versus ω-7 FA accumulation (as mol %).

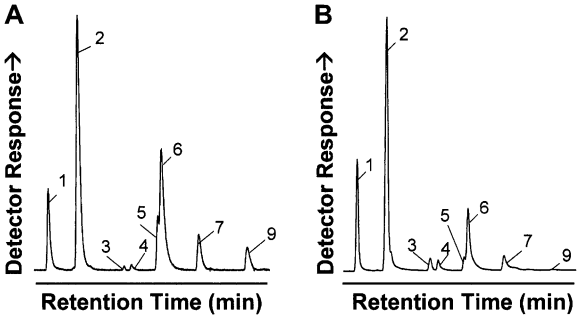

Figure 4. Representative chromatograms of FAMES from the best *fab1 fae1*, Phas:*Com25*, *Fab1*-HPAS, AnD9D, and SnD9D transformant line (A) and *Doxantha* seed (B). Peak designations are as described in Figure 2.

resulting in a decrease of seed 16:0 from approximately 21% to approximately 11% (approximately the level seen in wild-type seeds) with a corresponding increase in 16:1Δ^9 from approximately 30% to approximately 43% (Table II). The *fae1* mutant is almost entirely devoid of 16:1Δ^9 elongation activity so that levels of 18:1Δ^{11} are unaffected in the host *fab1 fae1 Com25* line when transformed with *SnΔ9D* and *AnΔ9D* (approximately 25% and 23%, respectively). Coexpression of plastidial and extraplastidial desaturases yielded a mean accumulation of approximately 67% ω-7 FA, with individual lines showing >71% ω-7 FA (Fig. 4). Seeds of the highest ω-7 FA (Fig. 4). accumulating lines had equivalent oil content to those of the wild type, 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, a subset 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 I), has a k_{cat} for 16:0 that is 135-fold lower than the approximately

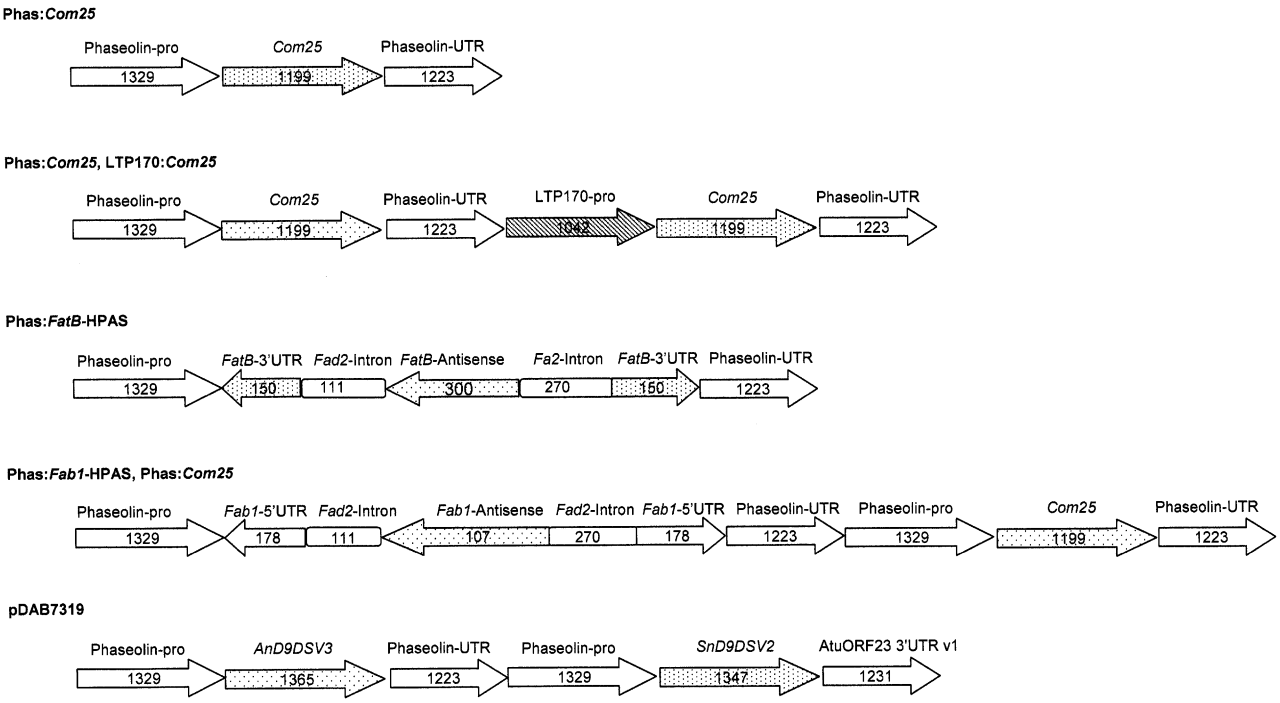

Figure 5. Schematic arrangement of DNA elements in constructs described in this article. Numbers indicate size in base pairs.

$1\ s^{-1}$ of the castor wild-type 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 wild-type desaturase for its natural 18:0 substrate. Thus, a key factor in the successful production of high levels of ω-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 ω-7 FA increases proportionately with 16:0 levels up to approximately 30% suggests that the 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 ω-7 FA was designed to mimic the strategy employed by naturally occurring ω-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). FAs 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 approximately 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 ω-7 monoene products to industrially relevant levels. Different strategies will likely be necessary to optimize the accumulation of oxygenated FAs, such as ricinoleic or vernolic acid (Lee et al., 1998), for which downstream enzymes, such as acyltransferases, with improved specificity for the unusual FAs appear to be important for improving the accumulation of the unusual FAs (Burgal et al., 2008). In another example involving the production of laurate in canola, the coexpression of a coconut (*Cocos nucifera*) 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, and (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 FAs.

Specifically, to optimize the accumulation of ω-7 FA we (1) introduced an engineered enzyme optimized for its specificity factor (k_{cat}/K_m) 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, and (3) increased product yield by expressing additional and compartment-specific 16:0-CoA desaturases.

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

MATERIALS AND METHODS

Arabidopsis Growth and Transformation

Arabidopsis (*Arabidopsis thaliana*) plants were grown in soil under continuous exposure to 300 μE of light (1 μE = 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 fluorescein isothiocyanate 535 and fluorescein isothiocyanate 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* Δ^9-18:0-ACP desaturase that arose from a program of combinatorial saturation mutagenesis/selection designed to identify variants with improved activity toward acyl chains of <18C in length (Whittle and Shanklin, 2001). *Com25* differs from the parental castor desaturase at the following 5 amino acid positions: M114S, T117R, L118C, P179L, and G188T (numbered according to the mature castor desaturase Protein Databank 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′ *Pac*I and 3′ *Xho*I restriction sites, was cloned into the corresponding sites of plasmid pDs-Red-Phas (Pidkowich et al., 2007; with Ds-Red marker) to create 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 afterward 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′ untranslated region was amplified from genomic DNA in both sense (using primers FatB-hps-5′*Pst*I 5′-GGGCTGCAGAAACAA-GTTTCGGCCACCAACCC-3′ and FatB-hps-3′*Xho*I 5′-CCCCTCGAGACA-TCAGAATTCGTAATGAT-3′) and antisense (using primers FatB-hpa-5′*Nhe*I 5′-GGGGCTAGCAAGTTTCGGCCACCAACCC-3′ and FatB-hpa-3′PacI 5′-CCCTTAATTAAACATCAGAATTCGTAATGAT-3′) orientations. These fragments were restricted with *Pst*I/*Xho*I and *Nhe*I/PacI and used to replace the 5′ untranslated region 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 (5′-CCACTAGTGGATCCACCTCTGCTACGTCGTCATT-3′) and FatB-Exon-3′Bg-Sal (5′-GGAGATCTGTCGACGTAGTTATAGCAGCAA-GAAG-3′), and the fragment, restricted with *Bam*HI and *Sal*I, was used to replace part of the *Fad2* intron after restriction with *Bgl*II and *Spe*I to create pGEM-T-Easy-HTM5.

The assembled HPAS fragment was excised with the use of *Pac*I and *Xho*I 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 was replaced by a green fluorescent protein marker pCVMV:Zs-Green [Clontech]) to create plasmid Phas:*FatB-HPAS* (Fig. 5).

Phas:*An*Δ9D and Phas:*Sn*Δ9D

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 *P. 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 *A. 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* and Phas:*Com25* to combine Com25 expression with KASII suppression. To achieve this, the *Eco*RV-*Eco*RV 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 *Eco*RI-*Eco*RI restriction sites and cloned into the corresponding site within Phas:*Fab1*-HPAS to create Phas:Fab1-HPAS-Phas-*Com25* (Fig. 5).

FA Analysis

To analyze the FAs of single seeds, we prepared fatty acid methyl esters (FAMEs) by incubating the seeds with 0.2 M trimethylsulfonium hydroxide in methanol (Butte et al., 1982). To similarly analyze bulk seeds, FAMEs were prepared by incubation in 0.5 mL BCl_3 for 1 h at 80°C, extracting them with 1 mL of hexane, and then drying under N_2. 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 × 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 monounsaturated FAMEs by dimethyl disulfide derivatization (Yamamoto et al., 1991).

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CORRECTIONS

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Mark S. Pidkowich was mistakenly omitted from the author list for this article. The author list should read as follows:

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