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Members of the Arabidopsis FAE1-like 3-Ketoacyl-CoA Synthase Gene Family Substitute for the Elop Proteins of *Saccharomyces cerevisiae*[*]

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Several 3-keto-synthases have been studied, including the soluble fatty acid synthases, those involved in polyketide synthesis, and the FAE1-like 3-ketoacyl-CoA synthases. All of these condensing enzymes have a common ancestor and an enzymatic mechanism that involves a catalytic triad consisting of Cys, His, and His/Asn. In contrast to the FAE1-like family of enzymes that mediate plant microsomal fatty acid elongation, the condensation step of elongation in animals and in fungi appears to be mediated by the Elop homologs. Curiously these proteins bear no resemblance to the well characterized 3-keto-synthases. There are three *ELO* genes in yeast that encode the homologous Elo1p, Elo2p, and Elo3p proteins. Elo2p and Elo3p are required for synthesis of the very long-chain fatty acids, and mutants lacking both Elo2p and Elo3p are inviable confirming that the very long-chain fatty acids are essential for cellular functions. In this study we show that heterologous expression of several *Arabidopsis* FAE1-like genes rescues the lethality of an *elo2*Δ*elo3*Δ yeast mutant. We further demonstrate that FAE1 acts in conjunction with the 3-keto and *trans*-2,3-enoyl reductases of the elongase system. These studies indicate that even though the plant-specific FAE1 family of condensing enzymes evolved independently of the Elop family of condensing enzymes, they utilize the same reductases and presumably dehydratase that the Elop proteins rely upon.

Cytosolic fatty acid synthases catalyze the *de novo* synthesis of the majority of cellular fatty acids, which have 16 or 18 carbons. However, there are many other types of fatty acids in the cellular lipids, including the very long-chain fatty acids (VLCFA)[2] that are synthesized through elongation of the C16 or C18 fatty acid by a microsomal enzyme system. The VLCFAs have been implicated in a number of cellular processes, including the formation of nuclear pores, trafficking of lipids and proteins, and the formation of membrane domains that organize signaling proteins. In higher plants, VLCFAs are critical components of the waxes found in the pollen coat and in leaves, and they are also found in the storage triacylglycerols of some seeds. Very long chain polyunsaturated fatty acids, including arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, are abundant in the membranes of brain and retina, and they serve as precursors of the biologically active eicosanoids. Despite mounting evidence that the VLCFAs are important in cellular physiology, many aspects of their synthesis and function remain to be resolved.

In yeast the saturated VLCFAs are present in the sphingolipids, which are essential for cell viability. Interestingly, a mutation that leads to the synthesis of C26-containing phosphatidylinositol can bypass the requirement for sphingolipids (though the mutant strains grow poorly) highlighting the importance of the VLCFA (1, 2). It is now well established that the Elop proteins are components of the membrane-associated elongase system that mediates VLCFA synthesis. Elo1p is involved in the elongation of medium-chain fatty acids, whereas Elo2p and Elo3p participate in the elongation of C16 to C24, and Elo3p is required for elongating C24 to C26 (3–6). Yeast transformed with foreign ELO homologs synthesize novel (*e.g.* mono- and polyunsaturated) elongated fatty acids demonstrating that the Elop proteins are responsible for selection of the acyl-CoA substrates for elongation (7–13). Although this implicates the Elop proteins as condensing enzymes, conclusive evidence that they directly catalyze the condensation reaction is lacking.

In plants the condensation step of fatty acid elongation is catalyzed by the FAE1-KCSs. For example FAE1, the founding member of the *Arabidopsis* FAE1-KCS gene family, is involved in the synthesis of the 22-carbon monounsaturated fatty acid (14). Lassner *et al.* (15) demonstrated that the *FAE1* homolog from jojoba encodes the condensing enzyme, 3-ketoacyl-CoA synthase (KCS), and Millar and Kunst (16) showed that KCS regulates the substrate specificity and activity of the elongation process. All 3-keto synthases characterized to date, including the FAE1-KCSs, the soluble fatty acid synthases, and those involved in polyketide synthesis, catalyze a Claissen condensation by essentially the same mechanism (17). In these systems, studies of the mechanism, analysis of essential catalytic residues, sequence homology, and, when available, the three-dimensional structure reveal that all of these condensing enzymes have a common ancestor.

In contrast, the Elop proteins provide an exception to the notion that all condensing enzymes involved in fatty acid elongation arose from a common origin. The hallmarks of all condensing enzymes studied to date are missing from the ELO gene family. For example, all condensing enzymes contain a conserved cysteine, which is the acyl acceptor in the first step of the mechanism. Although there are two conserved cysteines among the Elop homologs, there is no evidence of the catalytic triad that

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[2] The abbreviations used are: VLCFA, very long-chain fatty acids; KCS, 3-ketoacyl-CoA synthase; FAE-KCS, FAE1-like 3-ketoacyl-CoA synthase; FAMES, fatty acid methyl esters; IPC, inositolphosphoceramide; MIPC, mannosylinositolphosphoceramide; $M(IP)_2C$, mannosyldiinositolphosphoceramide; FOA, 5-fluoroorotic acid; GCMS, gas chromatography-mass spectrometry.

TABLE 1
Yeast strains used in this study

Strain name	Relevant genotype	Complete genotype
TDY2037	Wild type	Matα lys2 ura3–52 trp1Δ leu2Δ
TDY7000	***elo1*Δ**	Matα lys2 ura3–52 trp1Δ leu2Δ elo1::KAN
TDY7001	***elo2*Δ**	Mata lys2 his4 ura3–52 trp1Δ leu2Δ elo2::URA3
TDY7002	***elo3*Δ**	Matα ura3–52 trp1Δ leu2Δ elo3::TRP1
TDY7005	***elo2*Δ *elo3*Δ/pELO3**	Mata lys2 ura3–52 trp1Δ leu2Δ elo2::KAN elo3::TRP1/pRS316-ELO3
TDY7006	***elo1*Δ*elo2*Δ*elo3*Δ/pELO3**	Mata lys2 ura3–52 trp1Δ leu2Δ elo1::KAN elo2::TRP1 elo3::TRP1/pRS316-ELO3
TDY7007	***elo1*Δ*elo2*Δ*elo3*Δ/pFAE1**	Mata lys2 ura3–52 trp1Δ leu2Δ elo1::KAN elo2::TRP1 elo3::TRP1/pFAE1
TDY7010	***elo1*Δ*elo2*Δ*elo3*Δ/pAt1g04220**	Mata lys2 ura3–52 trp1Δ leu2Δ elo1::KAN elo2::TRP1 elo3::TRP1/pAt1g04220
TDY7009	***elo1*Δ*elo2*Δ*elo3*Δ/pAt5g43760**	Mata lys2 ura3–52 trp1Δ leu2Δ elo1::KAN elo2::TRP1 elo3::TRP1/pAt5g43760
TDY7008	***elo1*Δ*elo2*Δ*elo3*Δ/pAt16280**	Mata lys2 ura3–52 trp1Δ leu2Δ elo1::KAN elo2::TRP1 elo3::TRP1/pAt2g16280

TABLE 2
PCR primers used in this study

Primer name	Sequence
Elo2F	5′-GGCCGGATCCGTACGTATTCACATGTCCTG
Elo2R	5′-GGCCGGATCCTAGACATGACTGTCGAAAGG
Elo1F	5′-GGCATATGGGATCCTTCTGCCAGCCAACTCAAT
Elo1R	5′-GGCATATGGTCGACGTGTGACACTAGAATCGC
FAE1-PvuF	5′-CCGG CAGCTGATGACGTCCGTTAACGTTAAGCTCCTTTAC
FAE1-PvuR	5′-CCGG CAGCTGTTAGGACCGACCGTTTTGGACATG
FAE1-HAF	5′-GGGCCCCTCGAGCACGTCCGTTAACGTTAAG
FAE1-HAR	5′-GGGCCCCTCGAGTTAGGACCGACCGTTTTG
At2g16280-HAF	5′-CCCGGGCTCGAGCGAAGCTGCTAATGAGCCT
At2g16280-HAR	5′-CCCGGGCTCGAGTCAGAAGTCGAGCTTAAC
At5g43760-HAF	5′-CCCGGGCTCGAGCAGCCATAACCAAAACCAA
At5g43760-HAR	5′-CCCGGGCTCGAGCTACGACGATGTAATGGG
At1g04220-HAF	5′-CCCGGGCTCGAGCAATGAGAATCACATTCAA
At1g04220-HAR	5′-CCCGGGCTCGAGCTATCGAGATTCCGAGGA
At2g26250-HAF	5′CCCGGGGTCGACCGGTAGATCCAACGAGCAA
At2g26250-HAR	5′CCCGGGGTCGACTTAGAGAGGCACAGGGTA
At1g01120-HAF	5′-CCCGGGCTCGAGCGAGAGAACAAACAGCATT
At1g01120-HAR	5′-CCCGGGCTCGAGTCATTGCACAACTTTAAC
At2g26640-HAF	5′-CCCGGGGTCGACCGATGTAGAGCAAAAGAAA
At2g26640-HAR	5′-CCCGGGGTCGACCTAGATTGTGGAGACCTT
At1g19440-HAF	5′-CCCGGGGTCGACCGACGGTGCCGGAGAATCA
At1g19440-HAR	5′-CCCGGGGTCGACCTAATAACTTAAAGTTAC
At1g25450-HAF	5′-CCCGGGGTCGACCTCTGATTTCTCGAGCTCC
At1g25450-HAR	5′-CCCGGGGTCGACTCATAGTTTAACAACTTC
159AvrF	5′-GGATCCACCATGCCTAGGACTTTTATGCAA
159AvrR	5′-TTGCATAAAAGTCCTAGGCATGGTGGATCC
At159AvrF	5′-GGATCCACCATGCCTAGGGAGATCTGCACT
At159AvrR	5′-AGTGCAGATCTCCCTAGGCATGGTGGATCC

is found in other 3-keto synthases. A highly conserved feature of the Elop sequences is a histidine box, suggestive of a metal binding site, yet no other condensing enzymes contain such a domain. The FAE-KCSs are membrane-bound condensing enzymes with two membrane-spanning domains at the N terminus (18). In contrast, hydropathy analyses of the Elop homologs predict the presence of 5–7 transmembrane domains spread between the N and C termini.

In addition to the Elop proteins, there are several other proteins that are required for fatty acid elongation. Each C_2 elongation is a four-step process consisting of a condensation-reduction-dehydration-reduction cycle (see Fig. 1). The yeast *YBR159w* and *TSC13* genes encode the 3-keto and 2,3-enoyl reductases of the elongation cycle (19–21). Consistent with early biochemical characterization of fatty acid elongation that suggested the elongase enzymes are organized in a complex (22), the Elop, Ybr159p, and Tsc13p elongase proteins coimmunoprecipitate with one another (20, 21). The dehydratase component of the elongase system has yet to be identified, and whether there are other proteins required is also not yet known.

Although yeast have no FAE-KCS homologs, several *Arabidopsis* FAE-KCSs have been expressed in yeast, and these heterologously expressed enzymes are able to direct the synthesis of novel fatty acids (23). For example, expression of FAE1 in yeast leads to accumulation of monounsaturated C22 erucic acid (18, 23–25). That the FAE-KCSs in yeast lead to the synthesis of novel elongated fatty acids implies the reconstitution of a heterologous elongase and raises several questions. For example, can the FAE-KCSs substitute for the yeast Elop proteins? Does the conversion of the 3-keto intermediates generated by the FAE-KCSs into elongated fatty acids depend on the other enzymatic activities of the elongase complex? If so, do the FAE-KCSs physically associate with the reductases? In the studies reported here, we have addressed these questions.

EXPERIMENTAL PROCEDURES

Media, Strains, and Genetic Manipulations—Yeast media were prepared, and cells were grown according to standard procedures (26). The yeast strains used in this study are listed in Table 1.

Disrupting the ELO1, ELO2, and ELO3 Genes—The construction of the disruption alleles with *TRP1*-replacing parts of the coding sequences of *ELO2* and *ELO3* was described previously (21). The *elo2*::*KAN* disrupting allele was PCR-amplified using genomic DNA prepared from the yeast knock out collection (Invitrogen) and primers, ELO2F and ELO2R (Table 2). To construct the *elo1*::*KAN*-disrupting allele, a PCR fragment extending from 400 bp upstream from the start codon to 300 bp downstream of the stop codon of *ELO1* was generated using the primer pair ELO1F and ELO1R (Table 2) and genomic DNA prepared from wild-type yeast (TDY2037, Table 1). The PCR fragment was digested with BamH1 and SalI and ligated between the BamH1 and XhoI sites of a Bluescript plasmid to create pBS-ELO1. The SpeI/SalI

fragment from pFA6a-KanMX6 (27) was substituted for the XhoI/NheI fragment of pBS-ELO1, thereby replacing codons 10–183 of *ELO1* with the kanamycin resistance marker. The pBS-ELO1::KAN plasmid was digested with BamH1 and KpnI to liberate the *elo1*::*KAN* fragment that was used to disrupt the *ELO1* gene.

Construction and Screening of an Arabidopsis Yeast Expression Library—Inflorescences along with small amounts of stems, leaves, and newly initiated siliques were collected from *Arabidopsis thaliana* Columbia-0 plants and used immediately for the construction of a cDNA library in a yeast expression vector. Total RNA was initially isolated from the plant material by using TRIzol reagent (Invitrogen), and poly(A)$^+$ RNA was enriched by two successive passes of the total RNA through oligo(dT) cellulose columns (Amersham Biosciences), according to protocols provided by the manufacturers. cDNA inserts with EcoRI and XhoI restriction sites on the 5′- and 3′-ends, respectively, were generated from 5 μg of poly(A)$^+$-enriched RNA by using a Uni-ZAP cDNA synthesis kit (Stratagene), essentially as described in the manufacturer's protocol, except that Superscript II reverse transcriptase (Invitrogen) was used for synthesis of first strand cDNA. Enrichment for cDNAs of between ~700 and 3000 bp was conducted with Sephacryl cDNA size fractionation columns (Invitrogen). Size-selected cDNAs with flanking EcoRI and XhoI restriction sites were ligated into the EcoRI and SalI restriction sites of a modified form of the pADH yeast expression vector (21). Prior to its use, an EcoRI site was introduced into the multicloning site of the pADH vector by mutation of the HindIII site immediately adjacent to the *ADH* promoter. Following incubation at 14 °C for 24 h, the ligation reaction was drop-dialyzed against water for 15 min on 0.025-μm pore size VSWP filter discs (Millipore) and transformed into *Escherichia coli* DH10B cells (Invitrogen) by electroporation. Transformed cells were grown with shaking (250 rpm) for 1 h at 37 °C in 5 ml of LB media without selection. The cells were grown for an additional 5 h with ampicillin (100 μg/ml) selection and were then inoculated into 250 ml of LB media with ampicillin selection. Following growth at 37 °C with shaking for 8 h, plasmid was isolated from harvested cells and used for yeast complementation studies. The plasmid library was transformed into strain TDY7005 (*elo2*Δ *elo3*Δ/p*ELO3*, see Table 1), and *LEU2*$^+$ prototrophic transformants were selected. The transformants (254,000) were screened for growth on FOA, and 15 were identified that were able to lose the *URA3*$^+$-marked plasmid that carried the *ELO3* gene. The *LEU2*$^+$-marked plasmids from these strains were recovered, and their ability to rescue the *elo2*Δ *elo3*Δ double mutant was confirmed. Sequence analysis revealed that 9 carried the *At5g43760* FAE-KCS cDNA, 5 carried the At2g16280 FAE-KCS cDNA, and 1 carried the At1g04220 FAE-KCS gene.

Construction of the FAE1-KCS Expression Plasmid, the HA-FAE-KCS Expression Plasmids, and the Myc-tagged Yeast and Arabidopsis Ybr159p—FAE1-KCS was PCR-amplified from a cDNA kindly provided by Dr. Ljerka Kunst (15) using primers FAE1-PvuF and FAE1-PvuR. The amplified fragment was restricted with PvuII and ligated into a HindIII-cut pADH plasmid after filling the 5′-protruding ends with DNA polymerase I. For HA tagging of the FAE-KCSs, XhoI-ended or SalI-ended PCR products were generated that extended from codon 2 through to the stop codon for each gene. The primers used are listed in Table 2 with the *Arabidopsis* At gene designator and either -HAF (for HA-tagging primer, forward) or -HAR (for HA-tagging primer, reverse). The PCR-generated fragments were restricted with either XhoI or Sal1and ligated into the SalI site of plasmid pADH-3X-HA (21) resulting in an in-frame fusion of the N-terminal triple-HA tag to each of the FAE-KCSs. For tagging of the yeast Ybr159p and the *Arabidopsis* homolog of Ybr159p (encoded by *At1g67730* and hereafter referred to as AtYbr159p), a triple-Myc epitope tag was introduced at the N terminus of the pESC-Ybr159 and pESC-AtYbr159 constructs described before (17). For this an AvrII site was first introduced after the start codon in both constructs by QuikChange PCR mutagenesis (Stratagene), using the complementary mutagenic primers 159AvrF and 159AvrR or At159AvrF and At159AvrR, respectively. An SpeI-ended fragment carrying the triple-Myc cassette was generated by PCR using a Bluescript-based 3X-Myc-containing plasmid (19) and ligated into the AvrII site.

Elongase Assays—Microsomes were prepared from exponentially growing cells. Cells were pelleted at 5000 × *g*, washed with water, and repelleted. The cell pellets were resuspended in TEGM (0.05 M Tris (7.5), 1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin) buffer at 1 ml/50 OD cells, and glass beads (0.5-mm diameter) were added to the meniscus. Cells were disrupted by four cycles of vortexing for 1 min followed by cooling on ice for 1 min, and pelleted at 8,000 × *g* for 10 min. The supernatant was transferred to a fresh tube and spun at 135,000 × *g* for 30 min. The resulting pellet was resuspended by Dounce homogenization in at least a 10× volume of TEGM buffer and repelleted at 135,000 × *g*. The final membrane pellet was resuspended in TEGM buffer containing 33% glycerol and stored at −80 °C.

Total elongase activity was measured in a volume of 200 μl containing 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 150 μM Triton X-100, 1 mM NADPH, 1 mM NADH, 10 mM β-mercaptoethanol, 1 mM dithiothreitol, 40 μM acyl-CoA acceptor, and 60 μM 2[^{14}C]malonyl-CoA (0.05 μCi/ml) at 37 °C. The reaction was initiated by the addition of 0.2 mg of microsomal protein. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad). For assays of the condensing activity, the NADPH/NADH was omitted. The reaction was terminated after 10 min by adding 200 μl of 5 M KOH/10% MeOH and heating at 80 °C for 1 h. Following addition of 200 μl of 10 N H_2SO_4, free fatty acids were recovered by two 1.5-ml extractions into hexane. Following evaporation of hexane, the fatty acid methyl esters (FAMES) were generated by dissolving in 1 ml of 1 M methanolic HCl and heating at 80 °C for 1 h. An equal volume of 0.15 M NaCl was added, and the FAMES were extracted into hexane and resolved by reverse phase C18-silica gel TLC using $CHCl_3$:MeOH:H_2O (5:15:1) as the developing solvent. The radiolabeled fatty acids were detected and quantified using the Cyclone Storage Phosphor System (Packard Instrument Company, Inc., Meriden, CT).

Immunoblotting and Immunoprecipitation—Microsomes were prepared from strains containing HA-tagged FAE1-KCS and the yeast Myc-tagged Tsc13p or the *Arabidopsis* homolog of Tsc13p (encoded by *At3g55360* and hereafter referred to as AtTsc13p) (21, 28) or the yeast and the *Arabidopsis* Myc-Ybr159p (described above). The microsomes were solubilized at 1 mg/ml with 0.1% sucrose monolaurate (Roche Diagnostics) for 10 min, and the high speed (135,000 × *g*, 30 min) supernatant was collected. The supernatant (100 μl) was incubated with 3 μl of the precipitating antibody for 1 h and then with 25 μl of protein A-Sepharose (125 mg/ml from Sigma) for 1 h. The precipitates were washed three times with 1 ml of TEGM buffer containing 0.1% sucrose monolaurate and resuspended in 100 μl of SDS loading buffer, and a 10-μl sample was subjected to SDS-PAGE on a 4–12% gradient gel. Following transfer of the separated proteins to nitrocellulose, the blots were blocked in 0.1 M Tris, 7.5, 0.15 M NaCl, 0.1% Tween 20, 5% dry milk. The Myc-Tsc13p was detected with horseradish peroxidase-conjugated monoclonal anti-Myc antibodies (Sigma) at 1/2500. HA-FAE1-KCS was detected using horseradish peroxidase-conjugated monoclonal anti-HA antibodies (from Roche Molecular Biochemicals) at 1/1000.

The bound antibodies were detected by the ECL Western blotting detection system (Amersham Biosciences).

Fatty Acid Analysis—Cells (5×10^8) in mid-logarithmic growth were harvested and resuspended in 100 μl of distilled H_2O along with C21 and C23 fatty acids as internal standards. Fatty acids were extracted, and the fatty acid methyl esters were prepared as previously described (21). Gas chromatography/mass spectrometry (GCMS) was performed using an HP-6890 Series GC system with a Supelcowax 10 column, coupled to an HP-5973 Mass Selective Detector.

IPC Analysis—Cells were grown in SD medium (0.67% yeast nitrogen base, 2% bacto-agar, 2% glucose) without inositol overnight and harvested during exponential growth phase. The cells were resuspended to 10 A_{600}/ml in SD without inositol, and 50 μCi/ml myo-[2-^{3}H]inositol (16 Ci/mmol, Amersham Biosciences) was added. After a 10-min labeling, the cells were diluted to 2 A_{600}/ml with SD media containing 30 μM unlabeled inositol and incubated for an additional 90 min. Sodium azide (4 mM final) was added, and the lipids were extracted by bead beating in $CHCl_3$:MeOH (1:1). The extraction was repeated, and the extracted material was combined, clarified by centrifugation, dried, and desalted using water saturated butanol. 250,000 cpm were spotted on TLC and developed using $CHCl_3$:MeOH:H_2O:AcOH (16:4:1.6:4). The TLC was dried, sprayed with EN^{3}HANCE (PerkinElmer Life Sciences), and visualized using Kodak XAR film.

RESULTS

The elo Mutants Are Affected in the Condensation Step of Fatty Acid Elongation—A number of studies are consistent with the conclusion that the Elop proteins participate specifically in the condensation step of fatty acid elongation. For example, many previous studies of the Elop family of proteins have shown that they control the substrate specificity of fatty acid elongation (reviewed in Ref. 29). To address this question further, the condensation step of fatty acid elongation was assayed *in vitro* using microsomes prepared from wild-type and *elo1*Δ, *elo2*Δ, and *elo3*Δ mutant yeast cells. The ability of the mutant microsomes to direct the elongation of various acyl-CoA acceptors was assessed by measuring incorporation of label from [^{14}C]malonyl-CoA into elongated fatty acids. By omitting NADPH from the assay, the condensation reaction can be assayed (Fig. 1).

As shown in Fig. 2, microsomes prepared from wild-type cells form the 3-keto condensation products by condensing malonyl-CoA with each of the acyl-CoA substrates when NADPH is omitted. For example, with the C14-CoA substrate the C16–3-keto product accumulates, with the C16-CoA substrate the C18–3-keto product accumulates, and so forth (Fig. 2*A*). In contrast, when NADPH is included in the *in vitro* assay, the whole series of elongated fatty acids with chain lengths up to C26 are generated (Fig. 2*A*). Assays of the condensation reaction using the microsomes from the *elo* mutants confirm that the Elop proteins participate in the condensation step of elongation. Previous *in vivo* studies have shown that Elo1p is required for elongation of myristate to palmitate (3, 5, 6). The *in vitro* assays reveal that the condensation step is specifically affected, because microsomes prepared from cells lacking Elo1p are unable to form the C16–3-keto product when C14-CoA is used as a substrate for the condensation reaction (Fig. 2*B*). With the *elo1*Δ mutant microsomes, the longer substrates (C16-CoA to C24-CoA) are converted to the 3-keto products by Elo2p and/or Elo3p. The *elo2*Δ mutant microsomes have low condensation activity toward the C16 and C18-CoA substrates (Fig. 2*C*). Finally, the *elo3*Δ mutant microsomes form very little C24–3-keto product with the C22-CoA substrate and no C26–3-keto product with the C24-CoA substrate (Fig. 2*D*). These data provide additional evidence that the Elop proteins are required for the condensation step of elongation.

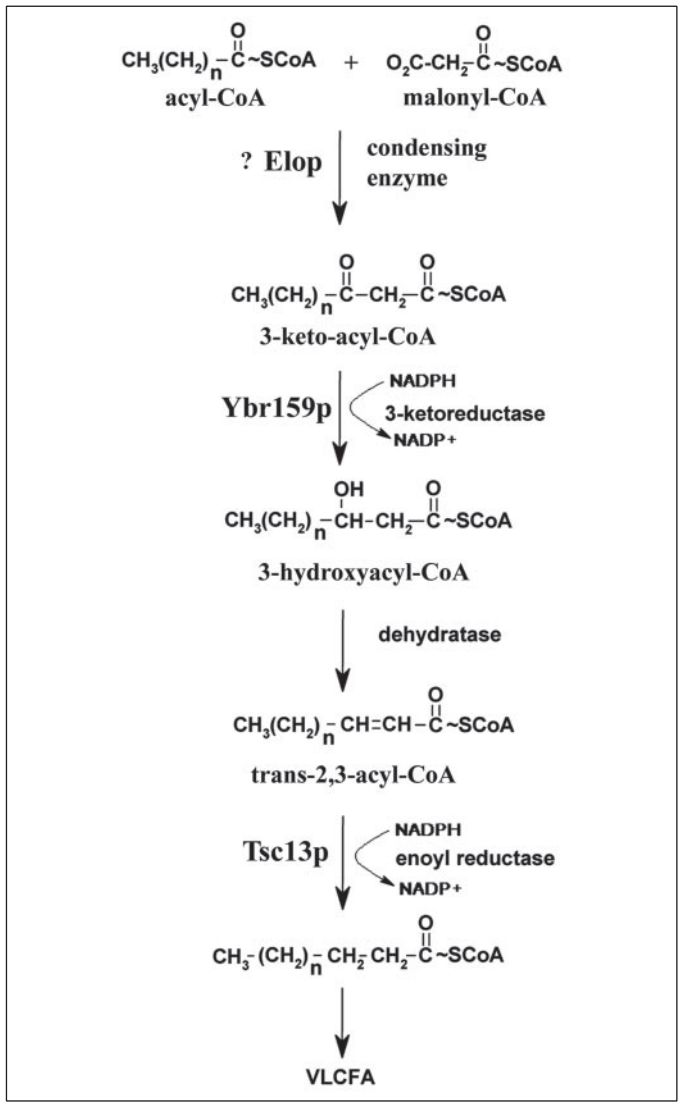


FIGURE 1. **Fatty acid elongation in *S. cerevisiae*.** Palmitoyl-CoA is elongated to a C26-VLCFA by the membrane-associated elongase enzymes. Each cycle of elongation involves four successive reactions, the condensation of malonyl-CoA with the acyl-CoA substrate, reduction of the 3-keto acyl-CoA, dehydration of the 3-hydroxy acyl-CoA, and reduction of the *trans*-2,3-acyl-CoA. Although the Elop proteins have not been shown to catalyze condensation, it is clear that they mediate substrate selection at the condensation step.

*The Arabidopsis FAE1 Gene and Other FAE-KCS Genes Rescue the Lethality of an elo2*Δ*elo3*Δ *Mutant*—The yeast *elo2*Δ*elo3*Δ double mutant is inviable, reflecting that the VLCFAs have essential functions for cell growth. Whereas the Elop proteins are needed for the condensation step of fatty acid elongation in yeast, members of the FAE1-like family of enzymes, hereafter referred to as the FAE-KCSs, catalyze the condensation step of fatty acid elongation in plants. As discussed above, there is no significant homology between the FAE-KCS proteins and the Elop proteins. Although previous studies have clearly demonstrated that *FAE1-KCS* expression in yeast leads to the synthesis of novel fatty acids (16, 18, 19, 23, 25), whether the FAE-KCSs can substitute for the yeast Elo2p/Elo3p proteins had not been tested. Therefore, it was of interest to test whether the *Arabidopsis* FAE1-KCS-condensing enzyme could rescue the lethality of the *elo2*Δ*elo3*Δ double mutant.

To address this question, a yeast mutant with chromosomal disruption of both the *ELO2* and *ELO3* genes that carried a wild-type *ELO3* gene on a *URA3*-marked, and therefore 5-fluoroorotic acid (FOA)-

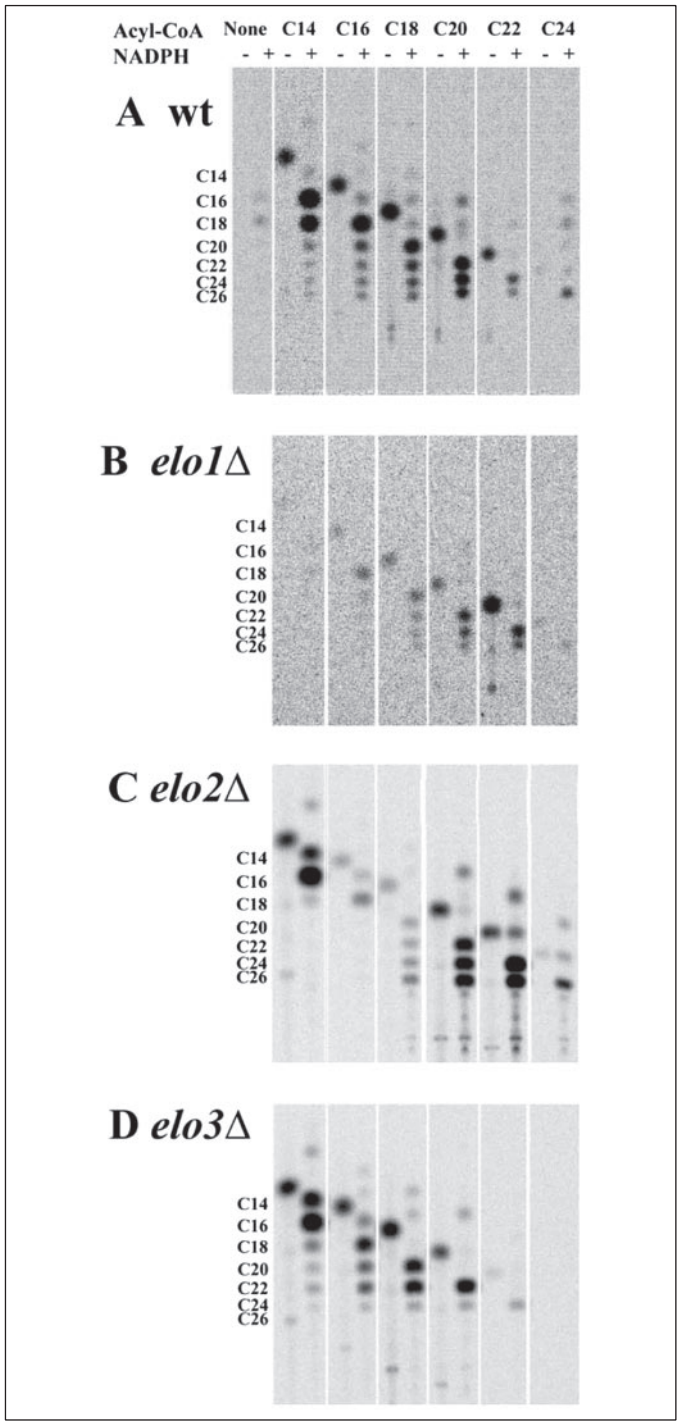


FIGURE 2. **The altered VLCFA composition of the *elo* mutants result from defects in the condensation step of fatty acid elongation.** Fatty acid elongation activity in wild-type (*A*), *elo1*Δ (*B*), *elo2*Δ (*C*), and *elo3*Δ (*D*) mutant microsomes was compared using C14-, C16-, C18-, C20-, C22-, and C24-CoAs as substrates. The assays were conducted in the absence of NADPH (−) to measure condensation activity and in the presence of NADPH (+) to measure overall elongation. The reaction products were converted to methyl esters, extracted, and separated by reverse-phase TLC. The chain lengths of the elongated products are indicated on the *left* of each panel. In the absence of NADPH, the 3-keto intermediate formed by the condensation of malonyl-CoA with the acyl-CoA acceptor accumulates. The radiolabeled spots present in the −NADPH lanes of the TLC are actually the 2-ketones that arise from decarboxylation of the 3-keto intermediates during the preparation of the FAMES (38). The small amount of C16 and C18 that forms in the absence of any added acyl-CoA (shown for the wt, labeled "*None*" in *panel A*) results from contaminating fatty acid synthase that purifies with the microsomes, as it is eliminated by including cerulenin in the assays or by deleting the *FAS2* gene.

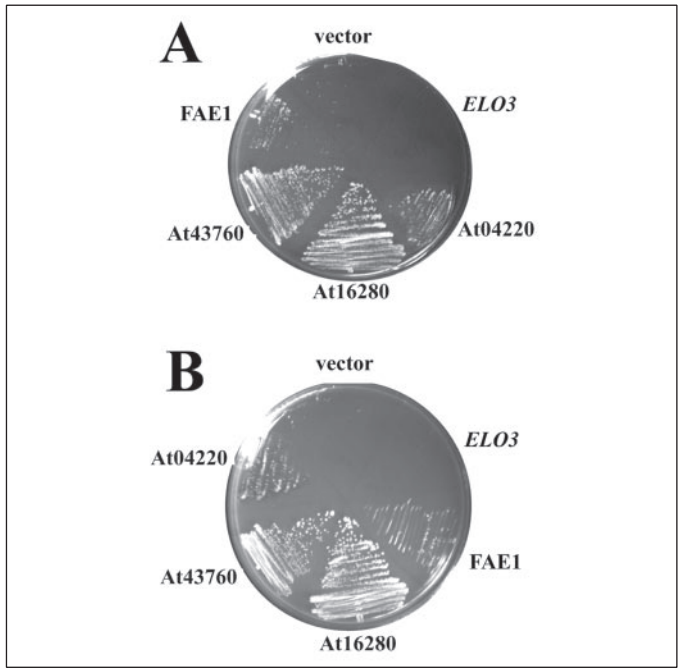
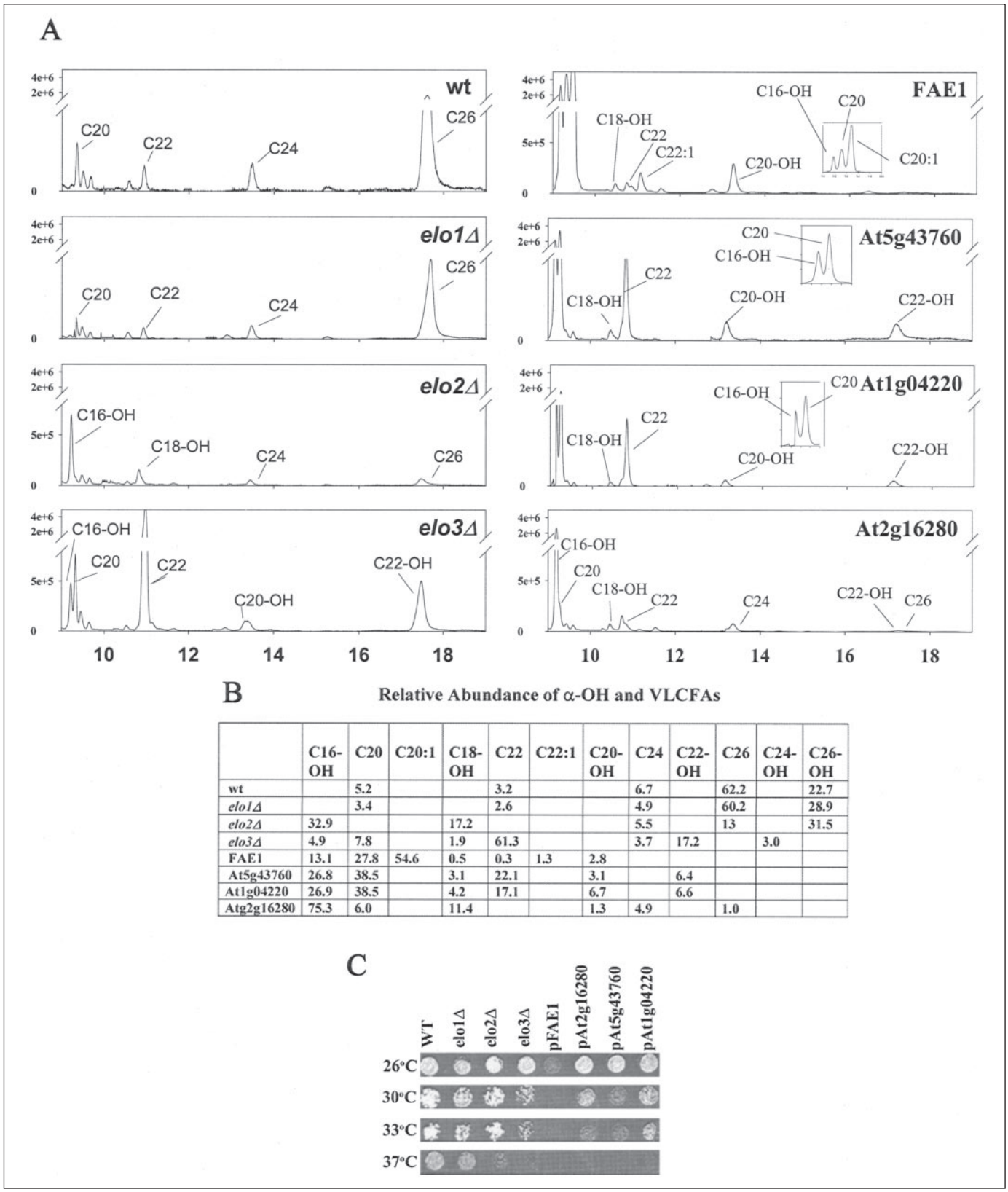


FIGURE 3. **Several *FAE-KCS* genes rescue the lethality of the yeast *elo2*Δ*elo3*Δ double mutant.** *A*, the *elo2*Δ*elo3*Δ double mutant is unable to lose the *URA3*-marked plasmid that carries the wild-type *ELO3* gene, and is therefore unable to grow on FOA. Introduction of the indicated *FAE-KCS* genes on a *LEU2*-marked yeast expression plasmid (but not the "*vector*" alone) allows the mutant strain to lose the *URA3*-marked *ELO3*-containing plasmid, demonstrating that the *FAE-KCS* genes can substitute for the *ELO3* gene. *B*, the *FAE-KCS* genes also rescue the *elo1*Δ*elo2*Δ*elo3*Δ triple mutant.

counterselectable, plasmid was used (TDY7005, *elo2*Δ::*KAN elo3*Δ::*TRP1/*p*ELO3*, Table 1). The *FAE1-KCS* cDNA was PCR-amplified and subcloned into a *LEU2*+-marked yeast expression plasmid ("Experimental Procedures"). Following transformation of the FAE1-KCS-encoding plasmid, the *elo2*Δ*elo3*Δ double mutant was able to lose the *URA3*-marked *ELO3*-rescuing plasmid (Fig. 3*A*). Consistent with the *in vitro* assays discussed above, knowledge that the FAE1-KCS condensing enzyme can rescue the *elo2*Δ*elo3*Δ double mutant provides additional evidence that the Elops are required specifically for the condensation step of elongation.

Whether other members of the *Arabidopsis* FAE-KCS gene family are able to restore viability to the *elo2*Δ*elo3*Δ double mutant was investigated by screening an *Arabidopsis* cDNA library. A yeast expression library was constructed by ligating *Arabidopsis* cDNAs behind the constitutively expressed yeast pADH1 promoter in a *LEU2*-marked plasmid ("Experimental Procedures"). The library was transformed into the *elo2*Δ*elo3*Δ double mutant carrying the wild-type *ELO3* gene on a *URA3*-marked plasmid (TDY7005, Table 1), and leucine prototrophic transformants were selected. Those transformants that were able to lose the *ELO3*-containing *URA3*-marked plasmid were identified by screening for growth on FOA. The cDNA-containing plasmids were recovered from the FOA-resistant transformants and retested to confirm that they were able to rescue the lethality of the *elo2*Δ*elo3*Δ double mutant. Three additional FAE-KCS genes, *At2g16280*, *At5g43760*, and *At1g04220*, were identified that rescue the *elo2*Δ*elo3*Δ mutant (Fig. 3*A*). It is likely that other members of the *Arabidopsis* FAE-KCS gene family can also substitute for the yeast Elo2p/3p proteins but that they are not as highly represented in this cDNA expression library. It is worth noting that five additional FAE-KCSs were PCR-amplified from the expression library, cloned into the 3X-HA expression vector, and confirmed to be expressed. However, they were not able to rescue the *elo2*Δ*elo3*Δ double mutant suggesting that they are unable to elongate the endogenous



B

Relative Abundance of α-OH and VLCFAs

	C16-OH	C20	C20:1	C18-OH	C22	C22:1	C20-OH	C24	C22-OH	C26	C24-OH	C26-OH
wt		5.2			3.2			6.7		62.2		22.7
elo1Δ		3.4			2.6			4.9		60.2		28.9
elo2Δ	32.9			17.2				5.5		13		31.5
elo3Δ	4.9	7.8		1.9	61.3			3.7	17.2		3.0	
FAE1	13.1	27.8	54.6	0.5	0.3	1.3	2.8					
At5g43760	26.8	38.5		3.1	22.1		3.1		6.4			
At1g04220	26.9	38.5		4.2	17.1		6.7		6.6			
Atg2g16280	75.3	6.0		11.4			1.3	4.9		1.0		

FIGURE 4. **The *FAE-KCS* genes restore VLCFA synthesis to the rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutants but do not completely reverse the phenotypes associated with VLCFA synthesis defects.** *A*, fatty acids were extracted from wild-type, *elo1*Δ, *elo2*Δ, *elo3*Δ, and *FAE-KCS*-rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutant cells, converted to methyl esters, and analyzed by GCMS. C21/23 fatty acids were added to the cells as internal standards prior to extraction of the fatty acids and were used for normalization but are not shown in the traces. The majority of the intracellular fatty acids are saturated and monounsaturated C16 and C18 (not shown in these traces) with the elongated (>C18) saturated fatty acids representing only ~5% of the total fatty acids (21). In wild-type yeast a high proportion (>50%) of the C26 is α-hydroxylated, also not shown in these traces. The *insets* identify the α-OH-C16, C20:0, and the C20:1 species. *B*, the relative abundance of the unhydroxylated saturated and monounsaturated C20–C26 and hydroxylated C16–C26 fatty acids is presented. *C*, the indicated strains were grown to an A_{600} of 0.5 and were diluted (1/5000) into the wells of a microtiter plate. The cells were transferred to plates containing YPD medium (1% yeast extract, 2% bacto-peptone, 2% bacto-agar, 2% glucose), and the plates were incubated at 26 °, 30 °, 33 °, or 37 °C for 3 days prior to photographing.

substrates to provide the saturated VLCFA required for yeast viability (see "Discussion").

Because Elo1p is involved in synthesizing C16 and C18 fatty acids, it was assumed that it was not required for the rescue of the *elo2*Δ*elo3*Δ double mutant by the FAE-KCSs. This was confirmed by demonstrating that an *elo1*Δ*elo2*Δ*elo3*Δ triple mutant (TDY7006, Table 1) harboring the FAE-KCS expression plasmids grew similarly to the rescued *elo2*Δ*elo3*Δ double mutant (Fig. 3*B*).

Characterization of in Vivo and in Vitro Activities of the FAE-KCSs—The elongated fatty acids that accumulated in the *elo2*Δ*elo3*Δ mutant rescued by the FAE-KCSs were analyzed by GCMS (Fig. 4*A*). The segment of the GCMS trace from 8 to 20 min profiling the elongated (>C18) and α-hydroxylated C16–C22 fatty acids is shown. Although these are the fatty acids relevant to this study, they represent a minor fraction (<5%) of the total fatty acids, with C16, C18, and C18:1 comprising the majority of the yeast fatty acids. In Fig. 4*B* the relative abundance of each of the elongated (>C18) and α-hydroxylated C16–C22 fatty acid species is indicated.

Consistent with previous characterizations (14, 19, 23), the predominant elongated saturated fatty acid in the FAE1-KCS rescued mutant is C20. In contrast to wild-type yeast, there are also significant levels of C20:1 and C22:1 in the *elo2*Δ*elo3*Δ mutant rescued by FAE1-KCS. This demonstrates that, unlike Elo2p and Elo3p, FAE1-KCS is able to elongate the C18:1 fatty acid that is present in yeast. The fatty acid profiles of the *At5g43760*- and *At1g04220*-rescued *elo2*Δ*elo3*Δ mutants are very similar, with the major VLCFA in these strains being C22. Overall, these rescued mutants have an *in vivo* fatty acid composition that is very similar to that of the *elo3*Δ mutant. The previously reported fatty acid profiles of yeast expressing these two FAE-KCSs (23) are somewhat different from the results reported here. In that study the genes were expressed in wild-type yeast, and thus the fatty acid profiles reflect the combined activities of the endogenous yeast Elo1/2/3 proteins and the heterologously expressed FAE-KCSs (see "Discussion"). The *elo2*Δ*elo3*Δ mutant rescued by *At2g16280* FAE-KCS accumulates significant levels of C24 VLCFA, making it the most similar to wild-type yeast.

Although they restored viability to the *elo2*Δ*elo3*Δ mutant, none of the heterologously expressed *FAE-KCS* genes restored wild-type VLCFA profiles. As mentioned above, the fatty acid profiles of the rescued mutants are similar to the fatty acid profiles observed in some of the yeast elongase mutants. For example, α-hydroxylated fatty acids with chain lengths of C16, C18, C20, and C22 accumulate, whereas in wild-type yeast only the α-hydroxylated fatty acid is the C26 species (Fig. 4*B*). From our previous studies (19–21), it is clear that the shorter α-hydroxylated fatty acids arise because phytosphingosine is acylated with shorter fatty acids in the VLCFA-deficient mutants, and the resulting C16–C22 ceramides are substrates for the Scs7p hydroxylase (30) (see "Discussion").

In addition to the altered VLCFA profiles, the FAE-KCS rescued *elo2*Δ*elo3*Δ mutants display other phenotypes that are characteristic of mutants with deficiencies in VLCFA synthesis. Similar to the *elo2*Δ and *elo3*Δ mutants, and in contrast to wild-type cells (20, 21), the rescued *elo2*Δ*elo3*Δ double mutants have high levels of the free long-chain bases (data not shown). These rescued strains also display temperature sensitivity, another common phenotype of the mutants with reduced VLCFA synthesis (Fig. 4*C*). The temperature-sensitive growth phenotype is most severe for the *FAE1-KCS*-rescued *elo2*Δ*elo3*Δ double mutant, which is not able to grow at or above 30 °C.

The analysis of the fatty acids that accumulate in the rescued mutants provides information about which of the endogenous yeast fatty acids can be elongated by the FAE-KCSs. To further investigate the activities

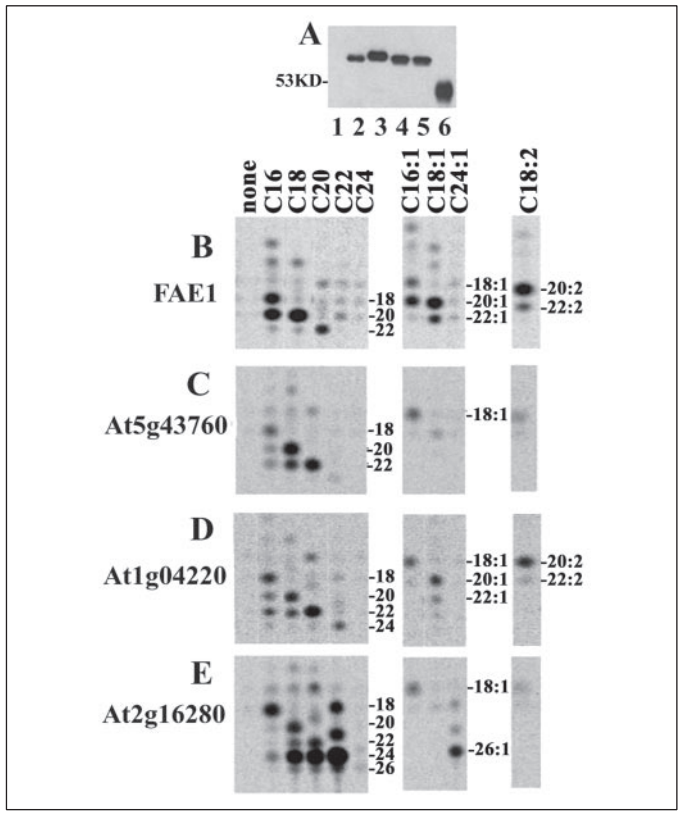


FIGURE 5. ***In vitro* elongase activities were measured using microsomes prepared from the *FAE-KCS*-rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutants.** *A*, the FAE-KCS proteins were expressed at similar levels. Microsomal protein (10 μg) was prepared from the *FAE-KCS*-rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutant and resolved by SDS-PAGE. The amino-terminally 3×-HA-tagged FAE-KCSs proteins (*lane 2*, HA-FAE1; *lane 3*, HA-At2g16280; *lane 4*, HA-*At5g43760*; *lane 5*, HA-At1g04220) were detected by immunoblotting with anti-HA antibody. The electrophoretic mobilities of the tagged FAE-KCSs were consistent with their predicted molecular masses (60–62 kDa). *Lane 1* contains protein from the mutant expressing no HA-tagged protein, and *lane 6* contains 3×-HA-tagged Elo3p (molecular mass of 43.7 kDa). *B–E*, fatty acid elongation activity was assayed using microsomes prepared from each of the FAE-KCS-rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutants. Microsomes were incubated with the indicated acyl-CoAs in the presence of NADPH, and the reaction products were analyzed by reverse phase TLC as described in Fig. 2. The chain lengths of the elongated products are indicated on the *right side* of each panel.

of the FAE-KCSs, *in vitro* elongase assays were conducted using microsomes prepared from yeast lacking the endogenous Elo1p/2p/3p proteins and rescued by each of the four *FAE-KCS* genes. Using a variety of different acyl-CoA acceptors, the substrate specificities of the FAE-KCS proteins were addressed. All four of these HA-tagged FAE-KCSs were expressed well in yeast, and similar amounts of the FAE-KCS proteins were present in the microsomal protein preparations used for these *in vitro* assays (Fig. 5*A*). The indicated acyl-CoAs were used at 40 μM in the standard elongation assay ("Experimental Procedures"). The elongase reactions were initiated by adding [^{14}C]malonyl-CoA along with NADPH, allowing overall elongation to be measured. The products were extracted and converted to the FAMES as previously described, and the FAMES were resolved by reverse-phase TLC (Fig. 5, *B–E*). In the reactions without added NADPH, substrates that could be elongated showed the expected 3-keto products (data not shown).

FAE1-KCS elongated the saturated C16- and C18-CoA substrates well, and also elongated C20–CoA to C22, albeit less well. The monounsaturated C16:1- and C18:1-CoA substrates were efficiently elongated, but FAE1-KCS had little or no activity toward the C22- and C24-CoAs or the C24:1-CoA. It also elongated the C18:2 substrate, forming C20:2 and small amounts of C22:2. The At5g43760 FAE-KCS displayed a strong preference for the C18- and C20-CoA substrates and

had activity toward the C16-CoA and the C16:1-CoA and very low activity with the C18:1-CoA (Fig. 5*C*). The At1g04220 FAE-KCS was similar to At5g43760 with activity toward the C16-, C18-, and C20-CoA substrates, but also had low activity toward C22-CoA. It also elongated the C16:1-, C18:1-, and C18:2-CoA substrates (Fig. 5*D*). The At2g16280 FAE-KCS utilized the C16–C22-CoAs and showed a strong preference for generating the C24 product, with very little of the intermediate C20 and C22 fatty acids accumulating (Fig. 5*E*). Interestingly, this FAE-KCS elongated the monounsaturated C24:1-CoA but displayed little activity toward the C16:1, C18:1, or C18:2 substrates. The spots above fully elongated products (especially apparent from the At2g16280-catalyzed reactions with the C20- and C22-CoA substrates) are most likely the 3-keto (seen in the reactions run without NADPH) and possibly the 3-hydroxy elongation intermediates.

The FAE-KCSs Coimmunoprecipitate with Ybr159p and Tsc13p, the Reductases of the Elongase Complex—The ability of the FAE-KCS enzymes to direct VLCFA synthesis in yeast raised questions as to whether this elongation pathway is dependent upon the other characterized components of the yeast Elop-containing elongase system. We have shown that Ybr159p is the major 3-keto reductase (19, 20) and that Tsc13p is the *trans*-2,3-enoyl reductase required for fatty acid elongation (21, 28). Furthermore, immunoprecipitation experiments have shown that these reductases, along with the Elop proteins, are organized in a complex. Because the FAE-KCSs are not homologous to the Elops, it was an open question as to whether they could associate with the other elongase proteins. However, the activity of FAE1 in yeast is dependent on Ybr159p (19), arguing that this component of the elongase is responsible for reducing the 3-keto intermediate generated by the FAE-KCSs. In addition, expression of the *FAE-KCS* genes did not bypass the slow growth phenotype of the *ybr159*Δ mutant or the lethality of the *tsc13*Δ mutant (data not shown).

Whether the FAE-KCSs physically associate with the reductases was tested directly by immunoprecipitation experiments. Membranes were prepared from cells coexpressing HA-tagged FAE1 with either Myc-tagged yeast or Myc-tagged *Arabidopsis* Tsc13p (Fig. 6*A*). Membranes were also prepared from cells co-expressing HA-tagged FAE1 with either Myc-tagged yeast or Myc-tagged *Arabidopsis* Ybr159p (Fig. 6*B*). Following solubilization, the microsomal proteins were immunoprecipitated with either HA or Myc antibodies. Samples of the solubilized membranes (Tot), along with the supernatants (Sup) and pellets (Pell) from the immunoprecipitations were immunoblotted with anti-HA or anti-Myc antibodies. The HA antibodies immunoprecipitated the Myc-tagged yeast Tsc13p and Myc-tagged *Arabidopsis* Tsc13p proteins along with the HA-tagged FAE1. Conversely, the Myc antibodies pulled down HA-tagged FAE1 along with the Myc-tagged yeast and *Arabidopsis* Tsc13p. The HA-tagged FAE1 also coimmunoprecipitated with Myc-tagged Ybr159p from *Arabidopsis* and to a lesser extent with Myc-tagged yeast Ybr159p (Fig. 6*B*). The weak interaction between FAE1 and yeast Ybr159p compared with its interaction with the *Arabidopsis* Ybr159p homolog is discussed further below ("Discussion"). Coimmunoprecipitation experiments using the HA-tagged At2g16280 FAE-KCS and Myc-tagged yeast and *Arabidopsis* Tsc13p confirmed that this FAE-KCS also associates with Tsc13p (Fig. 6*C*). These results demonstrate that the FAE-KCSs physically associate with the Tsc13p *trans*-2,3-enoyl reductase and the Ybr159p 3-keto reductase of the elongase system.

To determine whether FAE-KCSs and Elops can interact with the same elongase complexes, we also tested whether Elo3p coimmunoprecipitated with HA-FAE1 (Fig. 6*D*). The HA-FAE1 was expressed in wild-type yeast cells, and solubilized microsomal protein was immunoprecipitated with anti-HA-conjugated Sephadex beads. The failure to see any Elo3p (which migrates as three discrete bands due to differential glycosylation) coimmunoprecipitated with the HA-tagged FAE1 suggests that elongase complexes can form with either an Elop protein or a FAE-KCS protein, but not with both. The failure of Elo3p to coimmunoprecipitate with HA-FAE1 also demonstrates the specificity of the interactions of the FAE-KCSs with the reductases. It is important to note that the level of endogenously expressed Elo3p is higher than the levels of the HA-FAE-KCSs. This is evident from Fig. 5*A*, which shows that HA-Elo3p (expressed from its own promoter and thus reflecting endogenous Elo3p levels) is more abundant than the HA-tagged FAE-KCSs. The specificity of the association of the FAE-KCSs with the reductases is also underscored by the finding that an HA-tagged *Arabidopsis* Elop-like protein (At3g06460) failed to coimmunoprecipitate with Myc-tagged *Arabidopsis* or yeast Tsc13p (Fig. 6*E*). Finally, HA-tagged FAE1 also failed to coimmunoprecipitate with the endoplasmic reticulum-localized Lcb1p protein providing additional evidence for the specificity of the coimmunoprecipitations (data not shown). Taken together, these results strongly suggest that the FAE-KCSs associate specifically with the reductases of the elongase complex.

The VLCFA Synthesized by the Heterologously Expressed FAE-KCSs Are Incorporated into Inositolphosphoceramides—The experiments above demonstrate that the FAE-KCSs interact with the yeast elongase complex and direct the synthesis of sufficient VLCFA to rescue the lethality of an *elo2*Δ*elo3*Δ double mutant. In yeast, the majority of the VLCFAs are found in the sphingolipids, primarily the inositolphosphoceramides (IPCs). The IPCs are synthesized from the ceramides, which are in turn generated by joining the VLCFA in amide linkage with phytosphingosine in a reaction catalyzed by ceramide synthase (Fig. 7*A*). Because sphingolipids are essential for yeast cell viability, it appears that ceramide synthase is capable of using the VLCFA derived from the FAE-KCSs to acylate phytosphingosine, creating ceramides that can be converted to IPCs. The appearance of the α-OH C16–C22 fatty acids in the rescued mutants provides indirect evidence that the VLCFA produced by the FAE-KCSs are incorporated into ceramides, because Scs7p hydroxylates the fatty acid in ceramides. To directly test whether the FAE-KCS-derived VLCFAs are incorporated into sphingolipids, the FAE-KCS rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutant cells were labeled with inositol, and the extracted IPCs were analyzed by thin layer chromatography. As shown in Fig. 7*B*, each of the FAE-KCS-rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutants synthesized IPCs. Whereas the IPC in wild-type yeast contains exclusively α-OH C26 fatty acid (labeled as "*26*" Fig. 7*B*), mutants lacking Elo3p accumulate C22 IPC-C. The reduced hydrophobicity of the C22 IPC-C relative to the C26 PC-C is reflected in its lower mobility in the thin layer chromatographic analysis. Interestingly, the C22 IPC-C that accumulates in the *elo3*Δ mutant is not mannosylated; like the *csg* mutants that fail to convert IPC to MIPC (31, 32), the *elo3*Δ mutant makes IPC but no MIPC or $M(IP)_2C$ (Fig. 7*B*). With the exception of the strain expressing At2g16280, the IPCs that are synthesized by the FAE-KCS-rescued *elo1*Δ*elo2*Δ*elo3*Δ triple mutants are relatively hydrophilic and, like the C22 IPC-C that accumulates in the *elo3*Δ mutant, they are not mannosylated. The data are consistent with the fatty acid analyses (Figs. 4). For example, in the *FAE1*-rescued mutant, C20 is the major VLCFA in the cells and C20-IPC-C accumulates. Similarly, in the *At5g43760*- and At1g04420-rescued mutants, C22 is the predominant fatty acid and C22 IPC-C accumulates. The At2g16280-rescued mutant accumulates C24 and thus C24-IPC-C; unlike the IPCs with shorter fatty acids, this IPC species is mannosylated (note the appearance of MIPC). The spots above the indicated IPC-C species (and below PI) are IPC-B species that lack the α-hydroxylation on the fatty acids (30). The appearance of these IPC-B species in the

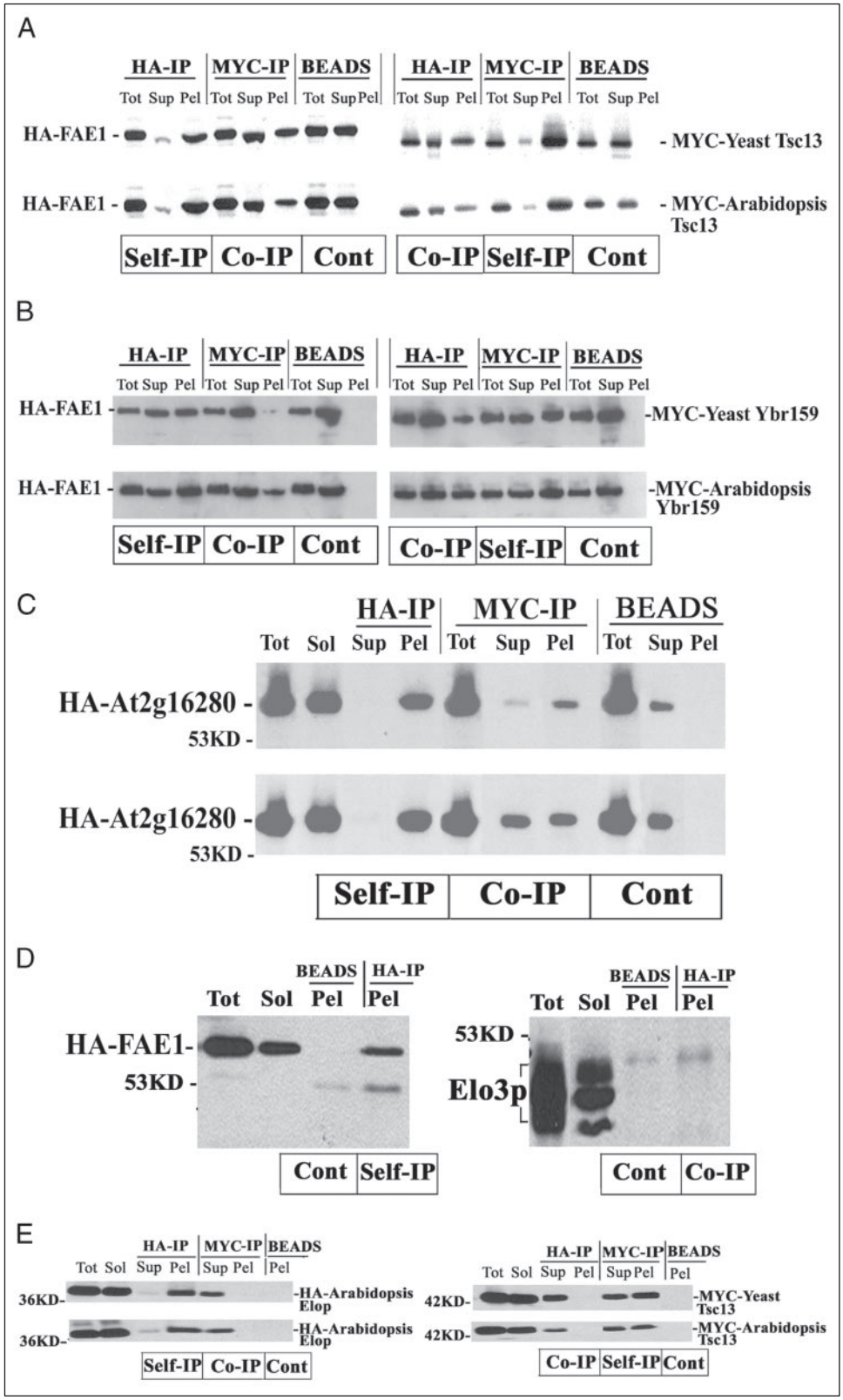


FIGURE 6. **FAE1 coimmunoprecipitates with the reductase components of the elongase complex.** *A*, HA-tagged FAE1 was co-expressed with Myc-tagged yeast Tsc13p (*top*) or Myc-tagged AtTsc13p encoded by the At3g55360 gene (*bottom*). Microsomes were prepared, solubilized, and immunoprecipitated with anti-HA antibodies, anti-Myc antibodies, or unconjugated Sephadex beads. The total soluble protein (*Tot*), supernatant (*Sup*), and pellet (*Pell*) from each immunoprecipitation was detected by immunoblotting with anti-HA (*left*) or anti-Myc (*right*). *B*, as in *A*, except that the HA-tagged FAE1 was coexpressed with Myc-tagged yeast Ybr159p (*top*) or Myc-tagged AtYbr159p encoded by the At1g67730 gene (*bottom*). The proteins migrate as predicted by their molecular masses, 41.4 kDa for yeast Myc-Tsc13p, 40.4 kDa for Myc-AtTsc13p, 38.7 kDa for yeast Myc-Ybr159p, 40.4 kDa for Myc-AtYbr159p, and 60 kDa for HA-FAE1. *C*, HA-tagged At2g16280 (62.2 kDa) was coexpressed with Myc-tagged yeast Tsc13p (*top*) or with Myc-tagged *Arabidopsis* Tsc13p (*bottom*). Microsomal protein (*Tot*) was solubilized (*Sol*) and immunoprecipitated with anti-HA or anti-Myc antibodies or with unconjugated Sephadex beads. The immunoprecipitates were immunoblotted with anti-HA antibodies. *D*, HA-tagged FAE1 was expressed in a wild-type yeast and immunoprecipitated with anti-HA antibodies or unconjugated Sephadex beads. Total microsomal protein (*Tot*) solubilized protein (*Sol*) and the pellets from the immunoprecipitations (unconjugated beads or anti-HA antibodies) were immunoblotted with anti-HA (*left*) or with anti-Elo3p (*right*). Elo3p, with a predicted molecular mass of 39.5 kDa, runs as a broad band due to glycosylation. *E*, the HA-tagged *Arabidopsis* Elop-like protein (At3g06460, 38.2 kDa) was coexpressed with Myc-tagged yeast Tsc13p (*top*) or with Myc-tagged *Arabidopsis* Tsc13p (*bottom*), and the solubilized proteins were immunoprecipitated with anti-HA or anti-Myc antibodies. The immunoprecipitates were immunoblotted with anti-HA (*left*) of anti-Myc (*right*) antibodies.

mutants that accumulate the C20 and C-22 IPCs suggests that the fatty acid chain length of the IPCs affects their hydroxylation by Scs7p.

DISCUSSION

The large number of different FAE-KCS isozymes (22 in *Arabidopsis*) is believed to reflect their activities toward different acyl-CoA substrates as well as the fact that some of the genes display tissue-specific expression. There are no *FAE-KCS* homologs in the yeast genome. Rather, the Elop family of proteins is believed to catalyze the condensation step of fatty acid elongation in yeast, as well as in other eukaryotes, including mammals. The evidence that the Elop proteins are responsible for condensation relies in part upon the finding that heterologously expressed

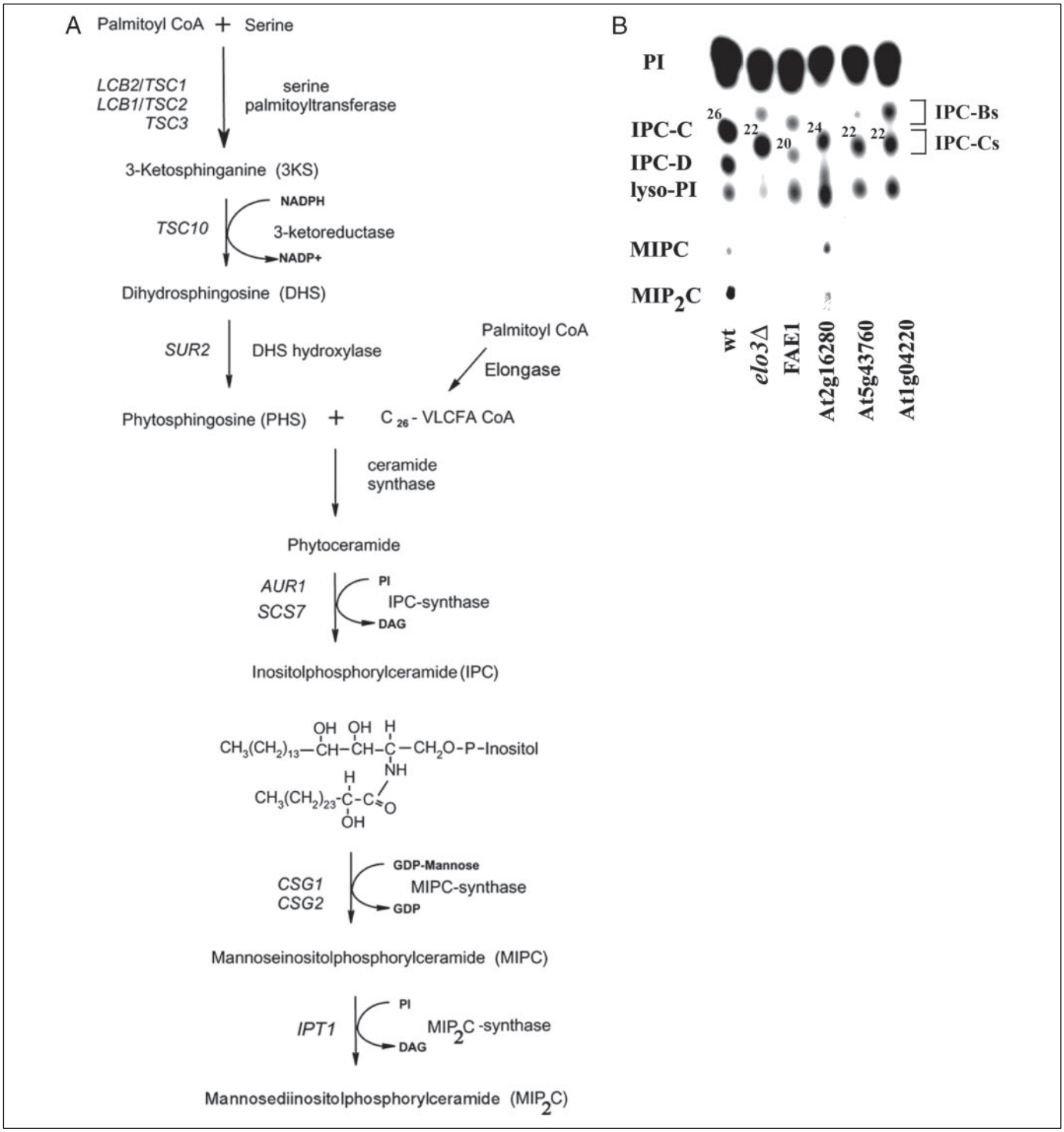


FIGURE 7. **The FAE-KCS-derived VLCFAs are incorporated into inositolphosphoceramides.** *A*, the pathway for synthesis of the IPC, MIPC, and MIP_2C sphingolipids in *S. cerevisiae*. *B*, wild-type, *elo3*Δ, and the *FAE-KCS*- rescued *elo1*Δ*elo2*Δ *elo3*Δ triple mutant cells were incubated with [^{3}H]inositol and the inositol-containing lipids were visualized by autoradiography following separation by TLC. Whereas the ceramide backbone of the IPC-C species in wild-type cells is comprised exclusively of PHS and an α-OH-C26 fatty acid, in the *FAE-KCS*-rescued mutants, IPCs with shorter chain lengths (C20–C24) are seen. The ceramides of the relatively hydrophobic IPC-Bs contain an unhydroxylated fatty acid. In addition to the inositolphosphoceramides, phosphatidylinositol (*PI*) as well as lyso-PI are present.

Elop homologs can direct the synthesis of novel fatty acids in yeast. Because the Elop proteins have none of the hallmarks of the well characterized 3-ketoacyl-CoA synthases, they either represent a completely novel type of condensing enzyme or they could be a regulatory subunit that confers acyl-CoA substrate recognition to an as yet unidentified condensing enzyme. In this regard, it should be noted that the dehydratase activity of the elongase system has not been identified, so it is entirely possible that additional elongase proteins will be identified.

We have recently reported that Ybr159p is the major 3-ketoreductase, and that Tsc13p is the *trans*-2,3-enoyl reductase of the yeast elongase system (19–21). Furthermore, we found that these enzymes coimmunoprecipitate with one another and with the Elop proteins, a result

that is consistent with early biochemical characterizations that suggested that the elongase enzymes are organized in a complex (33). Despite the lack of sequence homology between the Elop and FAE-KCS proteins, the data presented here demonstrate that the FAE-KCSs act in conjunction with the reductases of the elongase complex. The coimmunoprecipitation data strongly suggests that the FAE-KCSs physically associate in a complex with the Ybr159p and Tsc13p reductases. This raises the intriguing possibility that the Elop and FAE-KCS proteins evolved independently as distinct 3-keto-CoA synthases and yet are able to interact with the other components of the elongase complex. Although the significance is unclear, we previously observed that all of the known components of the yeast elongase complex (Elo1p/2p/3p, Ybr159p, and Tsc13p) have a high pI (> 10), and we note that the FAE-KCSs also have high pIs (>9.2). The coimmunoprecipitation results indicate that FAE1 interacts more strongly with the *Arabidopsis* Ybr159p homolog than with yeast Ybr159p. Although it is tempting to speculate that this reflects differences between the Ybr159p proteins that affect their association with FAE1, there is currently no information as to which proteins of the elongase complex are in direct contact.

In addition to the large FAE-KCS gene family, there are also several Elop homologs in *Arabidopsis*, the roles of which have not been defined. On the other hand, there is a single homolog of Tsc13p, the *trans*-2,3-enoyl reductase in *Arabidopsis* (encoded by *At3g55360* that functionally complements a yeast *tsc13*Δ mutant (28). Kunst and coworkers (34) recently reported the characterization of an *Arabidopsis* mutant lacking the Tsc13p homolog, referred to in that study as ECR. The mutant displayed reduced synthesis of VLCFA, and decreased cuticular wax, storage lipid, and VLCFA-containing sphingolipids. Although the plants displayed morphological abnormalities that could be specifically attributed to the sphingolipid deficiency, the plants were viable and accumulated some VLCFA, indicating that there is at least one other reductase that can reduce the *trans*-2,3-CoA intermediates in *Arabidopsis* (34). It is unclear whether the residual fatty acid elongation that is occurring in the mutant plants is dependent on the FAE-KCSs, or whether it might be mediated by the *Arabidopsis* Elop homologs.

There are two genes that encode 3-ketoacyl-CoA reductases with homology to Ybr159p in both *Arabidopsis* (*At1g67730* and *At1g24470*) and maize (*gl8a* and *gl8b*). The *gl8a* and *gl8b* genes of maize are 97% identical to one another, and both are required for normal accumulation of cuticular waxes. Double mutant kernels accumulate substantially reduced levels of VLCFAs and ceramides and are inviable suggesting that sphingolipids or other VLCFA-containing compounds are essential for normal embryo development (35). It is important to point out that the *Arabidopsis* reductase genes are able to substitute for their yeast counterparts (20, 21). Because yeast have no FAE-KCSs, these plant reductases clearly interface with the Elop proteins to restore fatty acid elongation in the yeast mutants. The role of the *Arabidopsis* ELOS has yet to be established, but it is likely that they function analogously to their yeast homologs and that the plant 3-keto and *trans*-2,3-enoyl reductases act with both FAES and ELOS in plants.

In this study four *Arabidopsis* FAE-KCSs that are able to substitute for the yeast Elo2p/Elo3p proteins by directing the synthesis of saturated VLCFA is reported. It is likely that additional members of the *Arabidopsis* FAE-KCSs will also functionally complement the *elo2*Δ*elo3*Δ double mutant, but that they were not recovered, because they are either not present or are poorly represented in the *Arabidopsis* yeast expression library. For example, despite the fact that *FAE1* does complement and that we were able to PCR amplify it from the expression library, it was not found in the complementation screening. The characterization of the complementing FAE-KCSs confirms that yeast require saturated VLCFA with a minimal chain length of C20 for viability. As mentioned under "Results," five additional FAE-KCSs (*At2g26250*, *At1g01120*, *At2g26640*, *At1g19440*, and *At1g25450*) were PCR-amplified from the library and expressed in yeast, but were unable to rescue the *elo2*Δ*elo3*Δ double mutant. Thus, they apparently cannot elongate the available substrates (mainly C16 in the *elo2*Δ*elo3*Δ mutant) to produce the required VLCFA. It will be interesting to characterize their substrate specificities using the *in vitro* elongase assays.

The heterologous expression of several *FAE-KCS* genes in wild-type yeast was recently reported (23), including several that were also characterized in this study. Three of the six that were found to be enzymatically active in that study (*FAE1*, *At5g43760*, and *At1g04220*) were among those that were found to be capable of complementing the *elo2*Δ*elo3*Δ double mutant. Expression of At1g01120 was reported to result in the accumulation of C22, -24, and -26 VLCFA and expression of *At1g25450* in the accumulation of C26, -28, and -30 (23), but we found that neither was able to rescue the *elo2*Δ*elo3*Δ double mutant. This may reflect the difference in expressing the genes in wild-type yeast instead of in the *elo2*Δ*elo3*Δ double mutant. For example, if these FAE-KCSs elongate fatty acids with chain lengths greater than C18, these substrates would have been available (produced by Elo2p/Elo3p) in a wild-type background but not in the *elo2*Δ*elo3*Δ double mutant background. Previous studies of yeast expressing the At1g01120 (*KCS1*) gene suggested that it does elongate C18, -20, -22, and -24 fatty acids (36). It is possible that the coexpression of some of these noncomplementing *FAE-KCSs* together with *FAE1* in the *elo2*Δ*elo3*Δ mutant will result in the ability to grow at 37 °C, a nonpermissive temperature for the FAE1-rescued mutant (Fig. 4*B*). For example, an FAE-KCS that elongates C20 substrates, along with FAE1 that elongates the C16 produced by *de novo* fatty acid synthesis to C20, would restore synthesis of VLCFA >C22 and thereby would likely restore growth at 37 °C.

Although this study confirms that the VLCFA produced by the FAE-KCSs fulfills the essential function missing in the *elo2*Δ*elo3*Δ mutant, the rescued strains are not completely normal. For example, the *FAE1-KCS*-rescued mutant is unable to grow at temperatures above 30 °C, a phenotype shared by a large number of mutants with reduced sphingolipid synthesis (37). This may reflect a role of the VLCFA-containing sphingolipids in reducing membrane fluidity at elevated temperatures. Furthermore, although only C26 ceramides are produced in wild-type yeast, the mutants accumulate ceramides with shorter chain lengths. Interestingly, the shorter ceramides are inositolphosphorylated by Aur1p, but the IPCs with fatty acids shorter than C24 are not substrates for mannosylation as no MIPC or $M(IP)_2C$ is generated.

In summary, we present here the finding that FAE-KCS proteins can substitute for the yeast Elop proteins. Surprisingly, although yeast have no FAE-KCS homologs, the FAE-KCS proteins apparently associate with the other components of the yeast elongase complex.

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