Molecular Characterization of the Fatty Alcohol Oxidation Pathway for Wax-Ester Mobilization in Germinated Jojoba Seeds[1[W]]

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Jojoba (*Simmondsia chinensis*) is the only plant species known to use liquid wax esters (WEs) as a primary seed storage reserve. Upon germination, WE hydrolysis releases very-long-chain fatty alcohols, which must be oxidized to fatty acids by the sequential action of a fatty alcohol oxidase (FAO) and a fatty aldehyde dehydrogenase (FADH) before they can be β-oxidized. Here, we describe the cloning and characterization of genes for each of these two activities. Jojoba FAO and FADH are 52% and 68% identical to Arabidopsis (*Arabidopsis thaliana*) FAO3 and ALDH3H1, respectively. The genes are expressed most strongly in the cotyledons of jojoba seedlings following germination, but transcripts can also be detected in vegetative tissues. Proteomic analysis indicated that the FAO and FADH proteins can be detected on wax bodies, but they localized to the endoplasmic reticulum when they were expressed as amino-terminal green fluorescent protein fusions in tobacco (*Nicotiana tabacum*) leaves. Recombinant jojoba FAO and FADH proteins are active on very-long-chain fatty alcohol and fatty aldehyde substrates, respectively, and have biochemical properties consistent with those previously reported in jojoba cotyledons. Coexpression of jojoba FAO and FADH in Arabidopsis enhanced the in vivo rate of fatty alcohol oxidation more than 4-fold. Taken together, our data suggest that jojoba FAO and FADH constitute the very-long-chain fatty alcohol oxidation pathway that is likely to be necessary for efficient WE mobilization following seed germination.

Wax esters (WEs) are oxoesters of long-chain fatty acids esterified with long-chain fatty alcohols. They have similar properties to triacylglycerols (TAGs) and are found widely in nature, where they have diverse and important biological functions (Hamilton, 1995). In higher plants, they are common components of the waxy cuticle on aerial surfaces (Samuels et al., 2008), but they generally do not accumulate to significant levels inside the cell. The only known exception is the jojoba plant (*Simmondsia chinensis*), which is a perennial woody shrub native to semiarid regions of southwestern North America (Moreau and Huang, 1981). Jojoba seeds contain large amounts of liquid WE that account for up to 60% of the dry weight of their cotyledons (Miwa, 1971) and that are stored in intracellular "wax bodies," which are approximately 1-μm-diameter lipid droplets with a structure analogous to that of conventional seed oil bodies (Moreau and Huang, 1977; Huang, 1992). These WEs are composed of very-long-chain (C20, C22, and C24) monounsaturated fatty acids and alcohols, and their physiological function is to serve as a primary storage reserve for postgerminative growth (Miwa, 1971).

Interestingly, jojoba WEs also possess unique physical properties that make them useful for a wide range of commercial applications, including cosmetic formulations, food products, and industrial lubricants (Lardizabal et al., 2000; Dyer et al., 2008). However, the agronomic performance of jojoba is poor compared with many "conventional" oilseed crops. To circumvent this, Lardizabal et al. (2000) successfully constructed the jojoba WE biosynthetic pathway in transgenic Arabidopsis (*Arabidopsis thaliana*), establishing that WEs can be made to accumulate in the seed. There has been considerable interest in refining this technology and translating it into a range of oilseed crops (Carlsson et al., 2011). It is conceivable, therefore, that any transgenic oilseed crops,

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which are modified to accumulate a high proportion of WEs in place of TAGs, may also require components of the jojoba WE mobilization pathway to allow for proper seedling establishment.

Huang and Moreau conducted a series of studies in the late 1970s to characterize the biochemical pathway for WE mobilization in jojoba cotyledons following seed germination (Moreau and Huang, 1977, 1979, 1981; Huang et al., 1978). These studies established that the majority of the metabolic pathway is shared in common with plant species that store TAGs in their seeds (Moreau and Huang, 1981; Graham, 2008). However, the initial conversion of WEs to fatty acids requires three activities that are potentially unique to jojoba (Fig. 1). WEs are hydrolyzed by a wax ester hydrolase (WEH; Enzyme Commission [EC] 3.1.1.50) that appears to be associated with the wax body surface to yield free fatty acids and alcohols (Huang et al., 1978). The alcohols are then oxidized to fatty acids by the sequential action of membrane-associated FAD-linked fatty alcohol oxidase (FAO; EC 1.1.3.20) and a NAD^+-dependent fatty aldehyde dehydrogenase (FADH; EC 1.2.1.48; Moreau and Huang, 1979).

While the molecular identities of the three enzymes are not known in jojoba, FAO and FADH genes have been characterized previously from a number of other organisms. For instance, FAO was first cloned in the yeast *Candida cloacae* (Vanhanen et al., 2000), and functional homologs have since been characterized in Arabidopsis and *Lotus japonicus* (Cheng et al., 2004; Zhao et al., 2008), although their precise physiological role(s) remains to be determined. FADH has been studied extensively in humans (*Homo sapiens*), where mutations in HsALDH3A2 are causal for the neurological disorder Sjögren-Larsson syndrome (De Laurenzi et al., 1996). The symptoms of this disorder (ichthyosis, spastic paraplegia, and mental retardation) are believed to arise from the accumulation of toxic levels of fatty alcohols and/or fatty aldehydes in the skin and brain (Rizzo, 2007). Homologs of HsALDH3A2 have been characterized in Arabidopsis (Stiti et al., 2011a), where loss- and gain-of-function studies suggest that they play a role in abiotic stress tolerance (Sunkar et al., 2003; Kotchoni et al., 2006; Stiti et al., 2011b). The aim of this study was to identify and characterize genes that are likely to be responsible for the very-long-chain FAO and FADH activities detected in jojoba cotyledons (Moreau and Huang, 1979) and to test whether these genes can form a functional fatty alcohol oxidation pathway in a heterologous oilseed host, such as Arabidopsis.

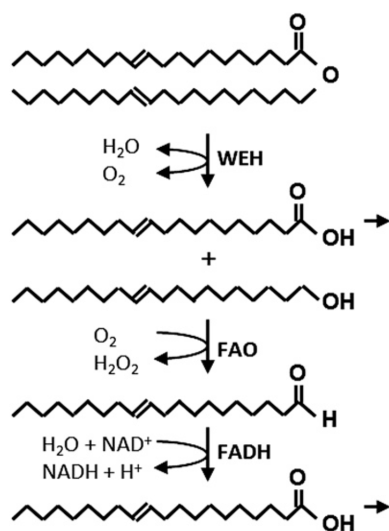

Figure 1. Schematic diagram of the pathway for WE mobilization in jojoba seeds. Horizontal arrows mark fatty acids destined for β-oxidation.

RESULTS

Cloning of FAO and FADH Genes from Jojoba

To identify genes that are associated with WE breakdown in jojoba, we took advantage of an EST library consisting of approximately 4,500 sequences from the cotyledons of germinated seeds (for details, see "Materials and Methods"). A TBLASTN search of this database identified several ESTs with similar sequences to alcohol oxidases and aldehyde dehydrogenases. However, two ESTs were selected for further analysis based on their greater similarity to the well-characterized archetypal FAO1 and ALDH3A2 proteins from *C. cloacae* and humans, respectively (De Laurenzi et al., 1996; Vanhanen et al., 2000). Gene-specific primers were designed against these EST sequences and were used to obtain full-length complementary DNAs (cDNAs) by 3′- and 5′-RACE. The sequences were designated ScFAO and ScFADH and were submitted to the GenBank database (accession nos. JX879776 and JX879777, respectively).

The deduced ScFAO protein has a predicted mass of 77 kD and, based on ClustalW2 amino acid sequence alignment (Larkin et al., 2007), is approximately 24% identical to *C. cloacae* FAO1 (Vanhanen et al., 2000) and approximately 52% identical to Arabidopsis FAO3 (At3g23410; Cheng et al., 2004; Supplemental Fig. S1). ScFAO also contains the five amino acid motifs that are proposed to be characteristic of the FAO protein family (Supplemental Fig. S1): the flavin-binding site (GXGXGG), Glc-methanol-choline oxidoreductase family signatures 1 (PROSITE PS00623) and 2 (PROSITE PS00624), the substrate-binding site, and the cytochrome *c* family heme-binding site (Cheng et al., 2004).

The deduced ScFADH protein has a predicted mass of 54 kD and is approximately 39% and 68% identical to human ALDH3A2 (De Laurenzi et al., 1996) and Arabidopsis ALDH3H1 (At1g44170; Stiti et al., 2011a), respectively (Supplemental Fig. S2). ScFADH contains the three amino acid motifs that are considered diagnostic of ALDHs (Supplemental Fig. S2): the ALDH Glu active-site signature sequence (PROSITE PS00687), the Rossmann

fold coenzyme-binding site (GXGXXG), and the catalytic thiol (PROSITE PS00070; Kirch et al., 2004). Notably, ScFADH, similar to HsALDH3A2, possesses a Val residue (Val-199) within the coenzyme-binding cleft and not an Ile residue, which Stiti et al. (2011a) recently showed was responsible for the strict NAD^+ dependence of AtALDH3H1 (Supplemental Fig. S2).

Transmembrane prediction tools, such as DAS (Cserzö et al., 1997) and HMMTOP (Tusnády and Simon, 2001), suggest that both ScFAO and ScFADH proteins have one or more membrane-spanning domains and therefore might be integral membrane proteins (Supplemental Fig. S3). ScFAO and ScFADH do not appear to contain a secretory sequence or known organelle-targeting sequences for the peroxisome, chloroplast, or mitochondria. However, both proteins contain a putative C-terminal di-Lys motif (Supplemental Figs. S1 and S2) typically found in endoplasmic reticulum (ER)-resident membrane proteins (Teasdale and Jackson, 1996; Gomord et al., 1999).

Jojoba FAO and FADH Are Most Strongly Expressed during Postgerminative Growth

Previous biochemical studies have shown that FAO and FADH activities are essentially absent from dry jojoba seeds and increase in the cotyledons following germination (Moreau and Huang, 1979). To investigate the expression patterns of ScFAO and ScFADH, quantitative reverse transcription (RT)-PCR was performed on RNA extracted from jojoba cotyledons over the course of germination and early seedling growth and also on RNA extracted from leaves, stems, and roots of 4-week-old plants (Fig. 2). The expression of both genes increased more than 20-fold in the cotyledons following germination, coinciding with the breakdown of WEs (Huang et al., 1978). Both genes were also expressed in the leaves, stems, and roots of jojoba plants; however, the levels of expression in these tissues were more than 10-fold lower than in the cotyledons (Fig. 2).

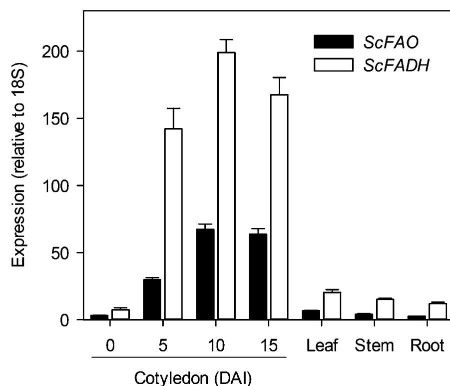

Figure 2. Quantitative PCR analysis of *ScFAO* and *ScFADH* gene expression in jojoba tissues. Values are means ± SE of measurements made on four separate RNA extractions and are normalized relative to *18S* expression. DAI, Days after seed imbibition.

Jojoba FAO and FADH Localize to the ER in Tobacco

Previous biochemical studies have shown that FAO and FADH activities are associated with the wax body and microsomal fractions in Suc gradients prepared from whole cell extracts of jojoba cotyledons (Moreau and Huang, 1979). Both ScFAO and ScFADH contain predicted membrane-spanning domains, and CcFAO1 and HsALDH3A2 are known to be membrane associated (Kelson et al., 1997; Vanhanen et al., 2000). Furthermore, ScFAO and ScFADH also contain putative di-Lys motif ER retention signals at their C termini (Supplemental Figs. S1 and S2). To investigate the subcellular localization of ScFAO and ScFADH, N-terminal GFP fusions were constructed and expressed transiently in tobacco (*Nicotiana tabacum* 'Bright Yellow-2' [BY-2]) suspension-cultured cells (Gidda et al., 2011) and leaf epidermal cells (Sparkes et al., 2006) under the control of the 35S promoter. Imaging of these cells using confocal laser-scanning microscopy showed that both GFP-ScFAO and GFP-ScFADH exhibit a subcellular localization pattern that is consistent with the ER and largely coincides with the ER marker stain concanvalin A in BY-2 cells and the ER marker protein signal sequence-red fluorescent protein (ss-RFP)-His-Asp-Glu-Leu (HDEL) in epidermal cells (Fig. 3). It is noteworthy that GFP-ScFAO also localizes to some regions that appear to be devoid of ER markers (arrows in Fig. 3).

FAO and FADH Can Be Detected in the Wax Body Fraction from Jojoba Seedlings

Although ScFAO and ScFADH localize to the ER when expressed as heterologous proteins in tobacco cells (Fig. 3), Moreau and Huang (1979) reported that FAO and FADH activity can be detected on wax bodies, as well as microsomal fractions, of jojoba seedling cotyledon homogenate. To investigate whether ScFAO and ScFADH are present on wax bodies, this fraction was prepared from crude cell homogenates of germinated jojoba cotyledons by flotation centrifugation (Huang et al., 1978), and the polypeptides were separated by SDS-PAGE (Eastmond, 2004; Supplemental Fig. S4A). The wax body fraction was highly enriched in low-molecular-mass polypeptides in the 15- to 26-kD range. This is a characteristic of seed oil bodies, which are coated in oleosins (Huang, 1992). Polypeptides in the 15- to 26-kD region were excised and subjected to tryptic digestion, and the resulting peptides were analyzed using nano-liquid chromatography-tandem mass spectrometry (MS/MS). No sequence information is currently available for jojoba oleosins. However, a Mascot search with the MS/MS data against the National Center for Biotechnology Information nonredundant database (http://www.matrixscience.com) provided significant ($P < 0.05$) matches with

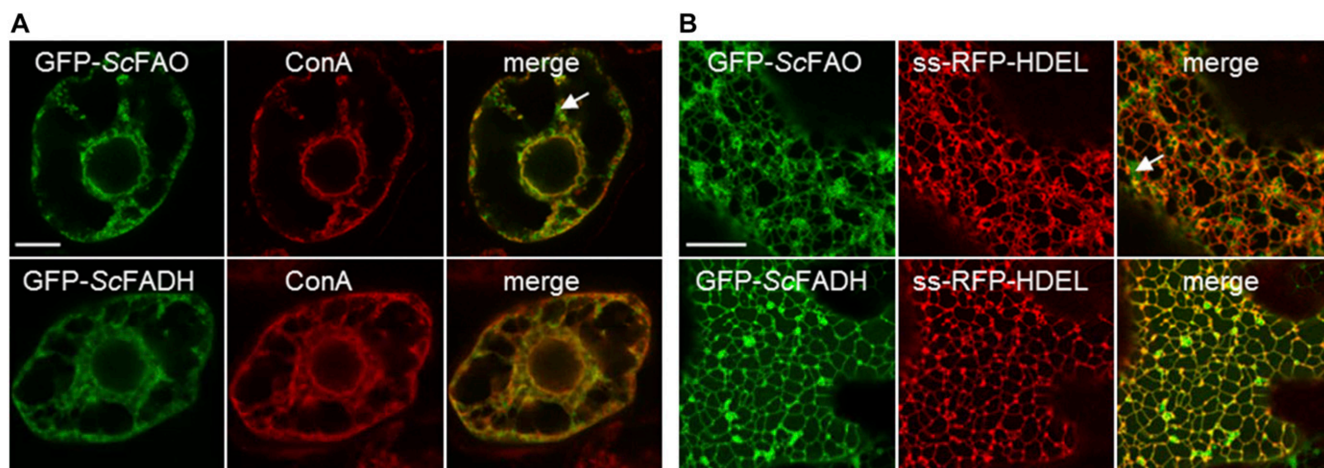

Figure 3. Subcellular localization of ScFAO and ScFADH. N-terminal GFP-tagged ScFAO and ScFADH were expressed transiently in tobacco BY-2 suspension-cultured cells (A) and leaf epidermal cells (B) under the control of the 35S promoter. Concanvalin A (ConA) and ss-RFP-HDEL were used to label the ER in BY-2 and epidermal cells, respectively. Arrows mark regions devoid of ER marker. Bars = 10 μm.

castor bean (*Ricinus communis*), grape (*Vitis vinifera*), and *Theobroma cacoa* oleosins (GenBank accession nos. XM_002516447, XP_002275496, and AAM46778, respectively). Lower abundance proteins with a molecular mass within the predicted range for ScFAO and ScFADH were then analyzed, and diagnostic mass fingerprints were identified for four peptides from ScFAO and seven peptides from ScFADH (Supplemental Fig. S4B), indicating that both proteins can be found in this subcellular fraction and therefore could account for the activity that has been detected there (Moreau and Huang, 1979).

Jojoba FAO and FADH Are Active on Very-Long-Chain Monounsaturated Substrates

We determined next whether ScFAO and ScFADH have very-long-chain alcohol oxidase and aldehyde dehydrogenase activities. Toward this end, His_6-tagged recombinant proteins were expressed in *Escherichia coli*, solubilized using Triton X-100, and affinity purified (Cheng et al., 2004; Stiti et al., 2011a). The FAO activity of recombinant ScFAO was measured using the spectrophotometric assay described by Cheng et al. (2004). Using dodecanol as a substrate, the pH optima was 8.5 to 9.0 and the K_m and V_{max} values were 42 μM and 0.12 μmol min^{-1} mg^{-1} protein, respectively (Table I). The chain-length specificity of ScFAO was determined using a range of commercially available saturated alcohols (C4–C18). No activity was detected using substrates shorter than C8, and activity was maximal between C10 and C14 (Fig. 4A). Jojoba WEs are composed almost exclusively of very-long-chain monounsaturated fatty alcohols (C20:1–C24:1). ScFAO was found to be active on these substrates when they were prepared from jojoba seeds and used in assays (Fig. 4A).

The ALDH activity of recombinant ScFADH was measured using the spectrophotometric assay described by Stiti et al. (2011a). Using dodecanal as a substrate and NAD^+ as a coenzyme, the pH optimum was 9.0 and the K_m and V_{max} values were 5 μM and 30 μmol min^{-1} mg^{-1} protein, respectively (Table I). A comparison of the kinetic constants for NAD^+ and $NADP^+$ showed that NAD^+ was the preferred coenzyme. Although ScFADH can use $NADP^+$, the relative catalytic efficiency was less than 1% of that for NAD^+, as deduced from the ratio $[V_{maxNAD^+}/K_{mNAD^+}]/[V_{maxNADP^+}/K_{mNADP^+}]$ (Table I). The chain-length specificity of ScFADH was determined using a range of commercially available saturated aldehydes (C3–C12) and NAD^+ as substrates (Fig. 4B). Propanal (C3) was a very poor substrate, while activity on dodecanal (C12) was greatest. ScFADH was also found to be active on very-long-chain monounsaturated fatty aldehydes (C20:1–C24:1) when these substrates were freshly prepared enzymatically from the alcohols using recombinant ScFAO and then provided in assays (Fig. 4B).

Expression of Jojoba FAO and FADH in Arabidopsis Enhances Fatty Alcohol Oxidation

To test whether ScFAO and ScFADH can constitute a functional fatty alcohol oxidation pathway in planta, the two proteins were constitutively expressed in Arabidopsis under the control of the 35S promoter. Two independent transformants with the highest expression level of each transgene, as determined by quantitative RT-PCR (Fig. 5A), were selected and crossed together. Homozygous lines carrying the single transgenes and both transgenes were then identified by segregation analysis. Five-day-old transgenic seedlings expressing ScFAO and ScFADH alone and in combination were

Table I. *Kinetic properties of recombinant enzymes*

Apparent K_m and V_{max} values were determined using affinity-purified recombinant ScFAO and ScFADH. Data represent means ± SE from at least three independent experiments.

Enzyme	Substrate	pH Optima	K_m	V_{max}
			μM	*μmol min^{-1} mg^{-1} protein*
ScFAO	Dodecanol	8.5–9.0	42 ± 3	0.12 ± 0.01
ScFADH	Dodecanal$_{(NAD^+)}$	9.0	5 ± 1	29.8 ± 3.0
ScFADH	$NAD^+_{(dodecanal)}$	9.0	450 ± 62	28.1 ± 4.2
ScFADH	$NADP^+_{(dodecanal)}$	9.0	2,371 ± 109	0.9 ± 0.2

incubated with 1-^{14}C-labeled octadecanol (C18), and oxidation was monitored by the rate of $^{14}CO_2$ release. In the wild-type control, significant $^{14}CO_2$ release was detected, showing that there is an endogenous capacity of fatty alcohol oxidation in Arabidopsis seedlings. However, transgenic seedlings expressing both ScFAO and ScFADH exhibited a rate of octadecanol oxidation that was up to 4-fold greater than in the wild type (Fig. 5). Seedlings expressing ScFAO alone had an approximately 2-fold higher rate of octadecanol oxidation, while expression of ScFADH resulted in an approximately 1.2-fold higher rate (Fig. 5). These data suggest that FAO activity is likely to be "rate limiting" for long-chain fatty alcohol oxidation but that enhanced FAO and FADH activity is required to achieve maximal rates of oxidation of this substrate.

DISCUSSION

Jojoba seeds are unusual in that their postgerminative growth is driven by the mobilization of WEs rather than TAGs (Miwa, 1971; Moreau and Huang, 1981). For every 1 mol of free fatty acid that is released by WE hydrolysis, 1 mol of fatty alcohol is also generated. Hence, there is an imperative for a highly active oxidation pathway capable of metabolizing the fatty alcohols to free fatty acids. Moreau and Huang (1979) described two activities from jojoba cotyledons that are likely to constitute this fatty alcohol oxidation pathway, and here we have identified and characterized the genes that encode enzymes with the appropriate properties. ScFAO and ScFADH were identified based on their high level of expression in jojoba cotyledons following seed germination and their homology to characterized FAOs and FADHs from other organisms. Recombinant ScFAO and ScFADH have enzymatic properties that are broadly consistent with those reported from jojoba cotyledon extracts (Moreau and Huang, 1979). Both proteins are active on commercial medium- and long-chain fatty alcohols and aldehydes, as has been reported for other FAO and FADH proteins (Kelson et al., 1997; Cheng et al., 2004; Eirich et al., 2004; Zhao et al., 2008; Stiti et al., 2011a). In addition, ScFAO and ScFADH can also use very-long-chain monounsaturated substrates, which would likely be their physiological substrates in jojoba. However, these substrates were not tested in the studies cited above; therefore, it is not clear whether the capacity to use very-long-chain monounsaturated substrates is specific to ScFAO and ScFADH. ScFAO is a FAD-linked enzyme that uses molecular oxygen as an electron donor and generates hydrogen peroxide. ScFADH is an NAD^+-dependent enzyme with comparatively low affinity for $NADP^+$. The K_m values for ScFADH using dodecanal and NAD^+ are also broadly similar to those reported by Moreau and Huang (1979). Both enzymes also have pH optima of approximately 9 and appear to be membrane bound.

Moreau and Huang (1979) reported that FAO and FADH activities in jojoba cotyledons are associated with the wax body fraction in Suc gradients prepared from whole cell extracts. However, a substantial amount of

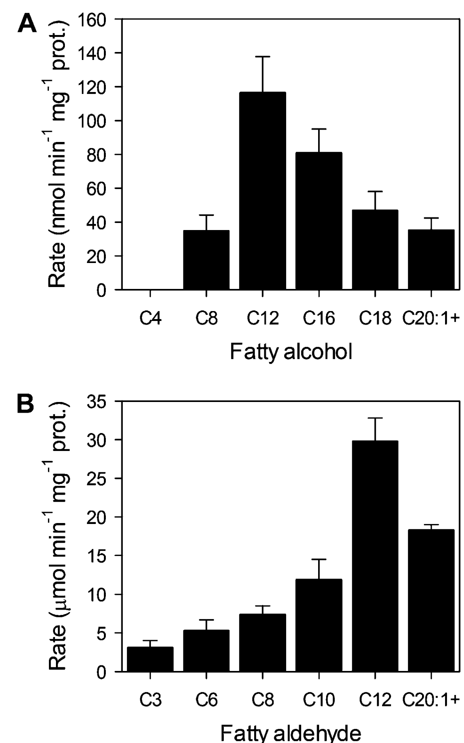

Figure 4. Substrate chain-length specificity of purified recombinant enzymes. Rates were determined for different alcohol and aldehyde substrates that were commercially available using affinity-purified ScFAO (A) and ScFADH (B). Jojoba fatty alcohols and aldehydes (C20:1+) consisting of a mixture of C20 to C24 monounsaturated acyl chains were also tested. Data represent means ± SE from at least three independent experiments.

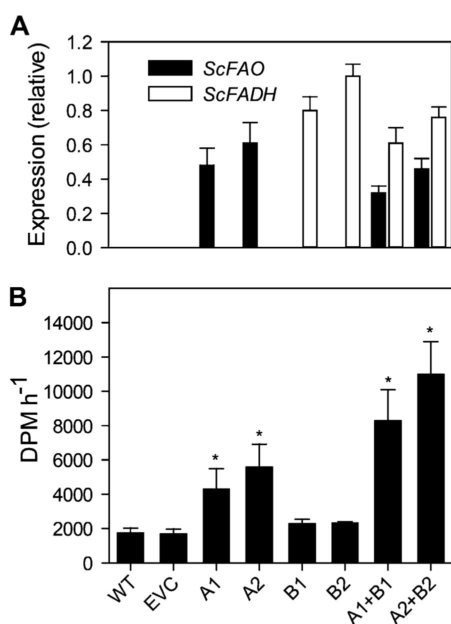

Figure 5. Effect of ScFAO and ScFADH expression on long-chain fatty alcohol oxidation by 5-d-old Arabidopsis seedlings. A, Quantitative RT-PCR analysis of transgene expression. Values are means ± SE of measurements on three batches of seedlings and are normalized relative to *18S* expression. B, Oxidation of long-chain fatty alcohol. Seedlings were incubated with 1-^{14}C-labeled octadecanol, and the initial rates of $^{14}CO_2$ release were monitored. WT, Wild type; EVC, empty vector control; FAO and FADH, homozygous lines expressing ScFAO and ScFADH, respectively. Numbers distinguish independent transformants, and + denotes lines expressing combinations of transgenes. Each incubation contained 20 seedlings, and values are means ± SE of four reactions. Asterisks denote significant differences from the wild type ($P < 0.05$).

activity was also present in the microsomal membrane fraction. ScFAO and ScFADH both contain a di-Lys motif at their C terminus, which may act as an ER retrieval signal (Teasdale and Jackson, 1996). When ScFAO and ScFADH were expressed individually in tobacco BY-2 or leaf epidermal cells as N-terminal GFP fusions, both proteins were targeted to the ER. This location is consistent with that of characterized homologs from yeast of the genus *Candida* and humans (Kelson et al., 1997; Vanhanen et al., 2000; Eirich et al., 2004). Arabidopsis FAO1 and ALDH3H1 have been also detected in ER-enriched cell fractions using mass spectroscopy (Dunkley et al., 2006). However, AtALDH3H1 has been shown to locate to the cytosol when expressed in Arabidopsis as a C-terminal fusion with GFP (Stiti et al., 2011b). ScFADH and AtALDH3H1 are quite closely related (68% amino acid identity), but AtALDH3H1 appears to lack a di-Lys motif; hence, the lack of this motif might explain the difference in subcellular localization. Alternatively, the apparent difference in ScFADH and AtALDH3H1 localization might be due to the GFP moiety being linked to the protein's N terminus (this study) versus C terminus (Stiti et al., 2011b). Whatever the reason, it is likely that the ER localization of GFP-ScFADH observed in this study reflects that of the native protein, because GFP would otherwise locate to the cytosol. It is noteworthy that ScFAO and ScFADH homologs have been identified from other plant species that contain a putative di-Lys ER retention signal, including AtFAO1 (Cheng et al., 2004), LjFAO2 (Zhao et al., 2008), soybean (*Glycine max*) ALDH3H2 (Kotchoni et al., 2012), and grape ALDH3J1 (Zhang et al., 2012).

It would be logical for the fatty alcohol oxidation pathway to be in close proximity to the wax body, since this is also the site of WE hydrolysis (Huang et al., 1978). Localization of ScFAO and ScFADH to the ER does not exclude the possibility that they may also associate with wax bodies. Several predicted membrane-spanning proteins from *Saccharomyces cerevisiae* have been reported to associate with lipid bodies as well as with the ER and other cellular membranes (Grillitsch et al., 2011). Consistent with this premise, we showed that ScFAO and ScFADH are associated with wax bodies prepared from homogenates of germinated jojoba cotyledons, as evidenced by the detection of diagnostic peptide mass fingerprints for both proteins in this fraction.

Although ScFAO and ScFADH exhibit the right biochemical attributes to account for very-long-chain fatty alcohol oxidation in jojoba cotyledons, the evidence we provide for their role is circumstantial. Unfortunately, jojoba is not practically amenable to loss- and gain-of-gene-function studies. However, to test whether the enzymes can form a fatty alcohol oxidation pathway, the two genes were expressed together in Arabidopsis, and radiolabeling experiments preformed on the resulting transgenic seedlings using a commercially available long-chain fatty alcohol as an exogenous substrate showed that the pathway was functional in planta. Specifically, we showed also that the introduced pathway requires both recombinant activities for maximum rates of oxidation, which were more than 4-fold greater than in the wild type. These data suggest that the two enzymes could potentially be useful in transgenic oilseed crops engineered to produce WEs (Carlsson et al., 2011), where the endogenous capacity for fatty alcohol oxidation might be insufficient to promote efficient reserve mobilization for postgerminative growth.

Interestingly, ScFAO and ScFADH are expressed in a range of jojoba tissues in addition to cotyledons. Indeed, homologs of both genes can be found in various higher plants, suggesting that they may be ubiquitously distributed throughout the plant kingdom. The physiological roles of these FAO and FADH genes in plants require further study. It is also conceivable that one or more of these enzymes might be involved in the synthesis of α,ω-dicarboxylic acids from ω-hydroxy fatty acids, both of which are common constituents of cutin and suberin (Pollard et al., 2008). ScFAO homologs from *Candida tropicalis* strain ATCC 20336 are capable of oxidizing ω-hydroxy fatty acids as well as fatty alcohols (Eirich et al., 2004). In vitro experiments actually lend

experimental support for an NADP$^+$-dependent pathway over a hypothetical alternative, catalyzed by a multifunctional cytochrome P450-dependent enzyme (Pollard et al., 2008). In Arabidopsis, there is also genetic evidence to show that a putative oxidoreductase gene called *HOTHEAD* (*HTH*) is responsible for converting ω-hydroxy fatty acids to ω-oxo fatty acids (Kurdyukov et al., 2006). HTH is related to *Candida* spp. FAOs (Kurdyukov et al., 2006), although not as closely as is ScFAO or the family of four FAOs identified in Arabidopsis by Cheng et al. (2004).

Another possible role for FAO and FADH isozymes is that they play a "housekeeping" role in fatty alcohol/aldehyde degradation in plant tissues. There may be a necessity to degrade fatty alcohols and fatty aldehydes as cytotoxic by-products of other cellular processes. In particular, oxidative stress leads to the generation of reactive aldehydes (Stiti et al., 2011a). Interestingly, AtALDH3H1, which is approximately 67% identical to ScFADH, is transcriptionally induced by abiotic stress and by abscisic acid (Kirch et al., 2005). Furthermore, loss- and gain-of-function studies suggest that Arabidopsis FADH isozymes play a role in abiotic stress tolerance (Sunkar et al., 2003; Kotchoni et al., 2006). A detoxification role of FADH is also well known in mammals (Rizzo, 2007). For instance, FADH deficiency in animal cells leads to the accumulation of fatty aldehydes and subsequently to Schiff's base formations with biologically important amines, such as those present in proteins and phospholipids (James and Zoeller, 1997; Rizzo, 2007). It has also been proposed that these unwanted modifications could be responsible for the symptoms of Sjögren-Larsson syndrome in humans (Rizzo, 2007). Long-chain fatty alcohols are major constituents of the epicuticular wax layer (Samuels et al., 2008). Remarkably, recent studies have indicated that the constituents of this layer (including fatty alcohols) are actively turned over and that neither chemical conversion within the epicuticular layer nor erosion/evaporation can account for their loss (Jetter and Schäffer, 2001; Gao et al., 2012). Therefore, it is possible that fatty alcohols, recycled from the epicuticular layer, are continually being degraded in epidermal cells of aerial plant tissues by an oxidation pathway that might rely on FAO and FADH.

In conclusion, our data suggest that ScFAO and ScFADH are very likely to constitute the fatty alcohol oxidation pathway that allows jojoba cotyledons to β-oxidize very-long-chain monounsaturated fatty alcohols that are releases by WE hydrolysis following seed germination (Huang et al., 1978; Moreau and Huang, 1979). The molecular identity of the jojoba WEH remains to be determined. It is known that several TAG lipases are capable of hydrolyzing WE in vitro (Tsujita et al., 1999). However, despite the wide distribution of WEs in nature (Hamilton, 1995), it is unclear whether a hydrolase has been cloned from any organism that has been shown to play a physiological role in this process. Further work will be required to clone and characterize the jojoba WEH.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Jojoba (*Simmondsia chinensis*) seeds were harvested from the field in Maricopa, Arizona. The seeds were imbibed in running water for 12 h and germinated on wet cotton wool in the dark at 30°C. Cotyledon samples were harvested at 0, 5, 10, and 15 d after imbibition for RNA extractions and at 10 d after imbibition for preparation of wax bodies. After 15 d, seedlings were transferred to constant light (300 μmol m^{-2} s^{-1} photosynthetic photon flux density) for a further 4 weeks before harvesting leaf, stem, and root samples for RNA extraction.

RNA Extraction, cDNA Cloning, RACE, and Quantitative PCR

DNase-treated total RNA was isolated from jojoba and Arabidopsis (*Arabidopsis thaliana*) tissues using either the RNeasy kit from Qiagen or the method of Wu et al. (2002). The synthesis of single-stranded cDNA was carried out using SuperScript II RNase H reverse transcriptase from Invitrogen. RACE was carried out using the GeneRacer Kit from Invitrogen following the manufacturer's instructions. A series of nested gene-specific primers for RACE were designed based on jojoba EST sequences provided by Prof. John Ohlrogge and Prof. Edgar Cahoon. The ESTs were part of a collection of approximately 4,500 sequences obtained from a germinated jojoba cotyledon cDNA library. Quantitative real-time PCR was performed with an ICycler (Bio-Rad) using qPCR Mastermix Plus (Eurogentec) according to the manufacturer's instructions, and the data were analyzed with the ICycler IQ5 software. The primer pairs used for real-time PCR were QScFAO (5′-TAACGACTCTCGATGGCAAG-3′ and 5′-ATATCGGGAGCCTGTGATTC-3′), QScFADH (5′-TGGAGAATCACGAGCAAGAG-3′ and 5′-GGATTCGAATTCAGGCTTGT-3′), QSc18S (5′-CGTTAACGAACGAGACCTCA-3′ and 5′-CCCAGAACATCTAAGGGCAT-3′), and QAt18S (5′-TCCTAGTAAGCGCGAGTCATC-3′ and 5′-CGAACACTTCACCGGATCAT-3′).

Heterologous Expression of ScFAO and ScFADH

ScFAO and ScFADH were PCR amplified from cDNA and subcloned into the appropriate vectors using standard molecular biology techniques. For expression in *Escherichia coli* strain BL21 (DE3), the pET28a expression vector (Novagen) was used to yield fusion proteins with an N-terminal His tag. Expression and purification of the His-tagged FAO and FADH proteins was performed according to the methods described by Stiti et al. (2011a) and Cheng et al. (2004), respectively. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard, and the purity of eluted proteins was verified by SDS-PAGE analysis (Cheng et al., 2004).

For expression in plants, ScFAO and ScFADH cDNAs were transferred to the binary vector pK7WGF2 (Karimi et al., 2002). Transient expression of the N-terminal GFP fusion proteins, and subsequent localization by confocal laser-scanning microscopy, were performed in tobacco (*Nicotiana tabacum* BY-2) suspension-cultured cells by biolistic bombardment (Gidda et al., 2011) and in leaf epidermal cells by *Agrobacterium tumefaciens*-mediated leaf infiltration (Sparkes et al., 2006). Details on the construction of ss-RFP-HDEL (encoding red fluorescent protein fused to the N-terminal Arabidopsis chitinase signal sequence and the C-terminal HDEL ER retrieval signal) and concanvalin A staining of BY-2 cells have been described previously (Shockey et al., 2006). The constructs were also transformed into Arabidopsis ecotype Columbia by the floral dip method (Clough and Bent, 1998). Positive transformants were selected via kanamycin resistance. Lines with the strongest expression of the transgene were identified by RT-PCR and crossed together to recover lines expressing both transgenes.

Enzyme Assays

Long-chain FAO activity was assayed by spectrophotometry using the method described by Cheng et al. (2004). The standard assay mixture contained 50 mM Tris-HCl (pH 8.5), 0.7 mg mL^{-1} 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 7 units of horseradish peroxidase, and substrate previously dissolved in acetone in a final volume of 1.0 mL. Reactions were initiated by the addition of enzyme, and the increase in A_{405} was measured. The value of the

extinction coefficient ε for the radical cation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) is 18.4 mM^{-1} cm^{-1}, and 1 mol of substrate gives rise to 2 mol of radical cation. No absorbance change was observed in the absence of either substrate of peroxidase. Long-chain FADH activity was measured using the spectrophotometric assay method of Kirch et al. (2001). The standard 1-mL reaction contained 100 mM sodium pyrophosphate (pH 9.0) plus NAD(P)$^+$ and substrate. Activity was monitored by the change in A_{340} following the conversion of NAD(P)$^+$ to NAD(P)H. The value for ε for NAD(P)H is 6.22 mM^{-1} cm^{-1}. Long-chain monounsaturated fatty alcohols and aldehydes were prepared from jojoba seeds for FAO and FADH assays. The alcohols were prepared from intact cotyledon tissue by transmethylation (Browse et al., 1986) followed by separation from fatty acid methyl esters using thin-layer chromatography (Lardizabal et al., 2000). The purified alcohols were used directly as substrate for FAO assays, and the fresh products of this assay were used as substrate for FADH assays.

Wax Body Purification and Proteomic Analysis

Wax bodies were separated from crude homogenate of germinated jojoba cotyledons by flotation centrifugation as described by Huang et al. (1978). Wax body proteins were then solubilized in SDS loading buffer and separated by SDS-PAGE (Eastmond, 2004). Polypeptides in the approximately 55- and 75-kD regions were excised in gel plugs, and destaining, digestion, and peptide extraction were performed by a MassPrep robotic protein-handling system (Micromass) using the manufacturer's protocol. The extracted tryptic peptides were resolved using an in-line NanoACQUITY ultra-performance liquid chromatography apparatus and Sample Manager System (Waters). A 4.9-μL aliquot of each sample was injected onto a nanoACQUITY UPLC Symmetry C18 trapping column, 5 μm, 180 μm × 20 mm (Waters), equilibrated in 1% aqueous acetonitrile containing 0.1% formic acid, and the column was flushed with 1% aqueous acetonitrile/0.1% formic acid at 15 μL min^{-1} for 1 min. The peptides were then eluted onto a nanoACQUITY UPLC PST C18 column, 1.7 μm, 100 μm × 100 mm (Waters), at 0.4 μL min^{-1} using a linear gradient (1%–50% buffer B) over 30 min with a total run time of 60 min. Buffer A was composed of 0.1% aqueous formic acid and buffer B was 0.1% formic acid in acetonitrile.

The eluted peptides were analyzed on a Q-ToF Ultima Global mass spectrometer (Waters) fitted with a fused silica emitter with an applied capillary voltage of 3.5 kV. The instrument was calibrated against a collisionally induced decomposition spectrum of the doubly charged precursor ion of [Glu^1] fibrinopeptide B. A calibration was accepted when the average error obtained on a subsequent acquisition was less than 10 ng g^{-1}. Sensitivity was assessed by an injection of 50 fmol of a glycogen phosphorylase B tryptic digest giving a base peak intensity of more than 1,000 counts s^{-1} in mass spectrometry mode on the most intense peptide. The instrument was operated in data-dependent acquisition mode over the mass/charge range of 50 to 1,950 with mass spectrometry and MS/MS data being collected from 10 min until 35 min of retention time. During the data-dependent acquisition analysis, tandem mass spectrometry (collisionally induced decomposition) was performed on the four most intense peptides as they eluted from the column. The uninterpreted MS/MS data were processed using the Waters ProteinLynx Global Server version 2.3 software package (smoothed, background subtracted, centered, and deisotoped) and mass corrected against the doubly charged [Glu^1] fibrinopeptide B peptide infused at 0.5 μL min^{-1} in 50% aqueous acetonitrile/0.1% formic acid through the nanolockspray line. Database search parameters specified trypsin as the digestion reagent, a mass tolerance of 50 ng g^{-1}, carbamidomethyl-Cys as a fixed modification, oxidized Met as a variable modification, with one peptide required for protein identification. The data were used to interrogate the jojoba EST database (plus ScFAO and ScFADH cDNA sequences) and the National Center for Biotechnology Information nonredundant database (version 20090926) using a MASCOT MS/MS search (http://www.matrixscience.com) to identify jojoba proteins from the tryptically digested gel bands.

Radiolabel Feeding Experiment

Five-day-old Arabidopsis seedlings were incubated at 20°C for 4 h in 500 μL of 50 mM MES-KOH (pH 5.2) containing 0.1 mM 1-^{14}C-labeled octadecanol (20 MBq mmol^{-1}) plus 1% (v/v) dimethyl sulfoxide. The reactions each contained 20 seedlings and were stopped by the addition of 0.5 mL of 6 M formic acid. Each reaction was conducted in a sealed 2-mL vial, and $^{14}CO_2$ was collected in a well within the vial containing 0.1 mL of 15% (w/v) KOH. The ^{14}C content of the KOH was determined by liquid scintillation counting.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JX879776 and JX879777.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. FAO polypeptide sequence alignments.

Supplemental Figure S2. FADH polypeptide sequence alignments.

Supplemental Figure S3. Transmembrane prediction results.

Supplemental Figure S4. Detection of polypeptides on wax bodies.

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