An Oleate Hydroxylase from the Fungus Claviceps purpurea: Cloning, Functional Analysis, and Expression in Arabidopsis

Dauenpen Meesapyodsuk and Xiao Qiu*

Department of Food and Bioproduct Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5A8 (D.M., X.Q.); and Bioriginal Food and Science Corporation, Saskatoon, Saskatchewan, Canada S7N 0W9 (D.M.)

Claviceps purpurea, a fungal pathogen responsible for ergot diseases in many agriculturally important cereal crops, produces high levels of ricinoleic acid (12-hydroxyoctadec-cis-9-enoic acid) in its sclerotia. It has been believed for many years that the biosynthesis of this fatty acid in C. purpurea involves a hydration process with linoleic acid as the substrate. Using degenerate polymerase chain reaction, we cloned a gene from the sclerotia encoding an enzyme (CpFAH) that has high sequence similarity to the C. purpurea oleate desaturase, but only low similarity to plant oleate hydroxylases. Functional analysis of CpFAH in yeast (Saccharomyces cerevisiae) indicated it acted predominantly as a hydroxylase, introducing hydroxyl groups at the 12-position of oleic acid and palmitoleic acid. As well, it showed Δ12 desaturase activities on 16C and 18C monounsaturated fatty acids and, to a much lesser extent, ω7 desaturase activities on ricinoleic acid. Heterologous expression of CpFAH under the guidance of a seed-specific promoter in Arabidopsis (Arabidopsis thaliana) wild-type and mutant (fad2/fad1) plants resulted in the accumulation of relatively higher levels of hydroxyl fatty acids in seeds. These data indicate that the biosynthesis of ricinoleic acid in C. purpurea is catalyzed by the fungal desaturase-like hydroxylase, and CpFAH, the first Δ12 oleate hydroxylase of nonplant origin, is a good candidate for the transgenic production of hydroxyl fatty acids in oilseed crops.
hydroxyl fatty acids at levels much lower than those found in native plant species (van de Loo et al., 1995; Broun and Somerville, 1997; Smith et al., 2003; Duak et al., 2007). This implies that additional genes from native species are required for the synthesis and accumulation of high levels of hydroxyl fatty acids in transgenic plants, and extensive efforts have recently been made to identify such genes (Kumar et al., 2006; Lu et al., 2006).

Claviceps purpurea, a fungal pathogen responsible for ergot diseases in plants, parasitizes young flowers of many grasses including agriculturally important cereal crops such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*; Scheffer and Tudzynski, 2006). During infection, this pathogen forms specialized structures called sclerotia in the ovaries of host plants. Although it has long been known that the sclerotia of this fungus produce high levels of ricinoleic acid (Mantle and Nisbet, 1976), the biosynthetic mechanism for fungal production of this fatty acid has not been established. Early reports proposed that biosynthesis of ricinoleic acid in *C. purpurea* was catalyzed by a hydrolase using linoleic acid as a substrate, and did not require molecular oxygen (Morris et al., 1966; Morris, 1970). This hypothesis has been held for many years. However, a recent study measuring site-specific deuterium abundance suggested that ricinoleic acid is formed in this species via a hydroxylase process, with oleic acid as the substrate (Billault et al., 2004).

Based on the recent results (Billault et al., 2004), we hypothesized that the biosynthesis of ricinoleic acid in *C. purpurea* might occur either by cytochrome P450-like hydroxylation or desaturase-like hydroxylation. In this study, we focused on the later possibility that the biosynthesis of ricinoleic acid in *C. purpurea* is catalyzed by a desaturase-like hydroxylase. By using degenerate RT-PCR targeted to conserved regions of fungal oleate desaturases, we cloned a cDNA from the sclerotium tissue of *C. purpurea* encoding an enzyme (CpFAH) with a high level of sequence similarity to fungal desaturases, and only a low level of similarity to plant fatty acid hydroxylases. Functional analysis of CpFAH in yeast (*Saccharomyces cerevisiae*) indicate its major activity is that of a hydroxylase, introducing a hydroxyl group at position 12 of oleic acid. Heterologous expression of CpFAH in Arabidopsis wild-type and mutant (*fad2/fae1*) plants revealed a higher level accumulation of hydroxy fatty acids in seeds than was previously observed in Arabidopsis transgenics carrying plant fatty acid hydroxylases.

RESULTS

Cloning of a *C. purpurea* Gene Encoding a Fatty Acid Hydroxylase

The fungal pathogen *C. purpurea* produces ricinoleic acid at high levels in its sclerotium tissue (Morris and Hall, 1966). To obtain this tissue, *C. purpurea* was grown in a sclerotium-inducing medium at room temperature for 4 weeks (Mantle and Nisbet, 1976). Fatty acid analysis of isolated fungal tissues indicated that sclerotium-forming mycelia obtained after 30 d of culture on sclerotium-inducing medium contained approximately 23% of the total fatty acids as ricinoleic acid, whereas this fatty acid was not detectable in non-sclerotium-forming mycelia.

To identify the gene encoding the hydroxylase involved in the synthesis of ricinoleic acid in *C. purpurea*, we designed several sets of degenerate oligonucleotide primers targeted to conserved domains of known fungal oleate desaturases. RT-PCR amplification with these degenerate primers, using total RNA isolated from sclerotium-forming mycelia as a template, generated three specific products, each showing sequence similarity to fungal Δ<sub>12</sub> desaturases.

Subsequently, full-length cDNAs corresponding to each of these products were obtained by 5′ and 3′ RACE. Sequence and functional analyses of full-length clones indicated that one cDNA, CpDes12, encodes a Δ<sub>12</sub> desaturase that introduces a Δ<sub>12</sub> double bond into oleic and palmitoleic acids. The second clone, CpDesX, encodes a novel desaturase catalyzing Δ<sub>12</sub>, Δ<sub>15</sub>, and ω<sub>3</sub> desaturation activity, with ω<sub>3</sub> desaturation of linoleic acid predominating (Meesapyodsuk et al., 2007). The third gene, CpFAH, which is characterized here, encodes a 477-amino acid polypeptide sharing high amino acid sequence similarity to CpDes12 (86% identity) and CpDesX (80% identity), as well as to fungal Δ<sub>12</sub> desaturases from *Fusarium* (68% identity) and *Aspergillus* (60% identity). Conversely, CpFAH shares a relatively lower level of sequence similarity with fatty acid hydroxylases from the plant species castor and *L. fendleri* (39%–40% amino acid identity).

Phylogenetic analysis of CpFAH and related enzymes shows that the three highly homologous enzymes from *C. purpurea*, CpFAH, CpDes12, and CpDesX, are grouped into a deep subbranch of the phylogenetic tree, belonging to a branch that includes other fungal Δ<sub>12</sub> desaturases (Damude et al., 2006; Fig. 1). Within this subbranch, CpFAH and CpDes12 are tightly grouped together. The fatty acid hydroxylases and Δ<sub>12</sub> desaturases from plants form a separate distant group in the phylogenetic tree.

Functional Characterization of CpFAH in Yeast

To determine the function of CpFAH, the coding region of the cDNA was cloned into the yeast expression vector pYES2.1 under the control of GAL1 promoter, and the recombinant plasmid was then introduced into the yeast strain INVSc1. Fatty acid analysis of total lipids indicated that yeast transformants expressing CpFAH (CpFAH/INVSc1) produced six new fatty acids compared to the control (pYES2.1/INVSc1; Fig. 2). Three of these fatty acids (peaks 1, 2, and 3) were identified as 16:2-9t,12c, 16:2-9c,12c, and 18:2-9c,12c, respectively, as previously described (Meesapyodsuk et al., 2007). Three new fatty acids
having longer retention times (peaks 4, 5, and 6) carried hydroxyl groups able to react with a trimethylsilylating reagent. The chemical structures of these hydroxyl fatty acids were determined by gas chromatography (GC)/mass spectrometry (MS) analysis of fatty acid methyl ester (FAME)-trimethylsilyl (TMS) derivatives. As shown in Figure 3A, the mass spectrum obtained from peak 5, including three diagnostic fragments at mass-to-charge ratio (m/z) 187 (A1), m/z 270 (A2), and m/z 299 (A3), is identical to that previously obtained for a TMS-methylricinoleate standard (van de Loo et al., 1995), indicating that this hydroxyl fatty acid is ricinoleic acid (12-OH-18:1-9c). Figure 3B shows the mass spectrum of FAME-TMS derivatives of peak 6. Two of the three diagnostic fragments, m/z 270 (B2) and m/z 299 (B3), are identical to fragment A2 and A3 of TMS-methylricinoleate, whereas fragment B1 differs from A1 of TMS-methylricinoleate by two mass units, which is due to the loss of two hydrogen atoms. Thus, this hydroxyl fatty acid is densipolic acid (12-OH-18:2-9c,15c), a fatty acid that was also observed as a product of plant hydroxylases (Broun and Somerville, 1997; Broun et al., 1998a). The mass spectrum of the peak 4 derivative is shown in Figure 3C. Two of the diagnostic fragment ions, C2 and C3, are identical to their counterparts A2 and A3 at m/z 270 and m/z 299. The diagnostic fragment ion C1 at m/z 159 differs from its counterpart A1 in the TMS-methylricinoleate spectrum by a reduction of 28 mass units, which corresponds to the mass of two carbon and four hydrogen atoms, indicating this new hydroxyl fatty acid is 12-OH-16:1-9c. This hydroxyl fatty acid was also observed when a Lesquerella oleate hydroxylase was expressed in yeast (Smith et al., 2003).

CpFAH expression led to the production of densipolic acid, which contains a ω3 double bond. Furthermore, when yeast transformants expressing CpFAH were supplied with linoleic acid (18:2-9c,12c), the increased linoleate levels resulted in the production of the ω3 desaturated product linolenic acid (18:3-9c,12c,15c; data not shown). Thus, the ω3 double bond in densipolic acid is likely derived from a minor ω3 desaturase activity of CpFAH on ricinoleic acid. The ability of ω3 desaturases to introduce a double bond at the ω3 position of ricinoleic acid has also been observed in plants (Meesapyodsuk et al., 2000; Reed et al., 2000). Among the six new fatty acids produced by CpFAH, ricinoleic acid was the most abundant, accounting for approximately 19% of the total fatty acids in transformed yeast cells. The fatty acids 16:2-9c,12c, 12-OH-16:1-9c, and 18:2-9c,12c were less abundant, accounting for approximately 8%, 4%, and 3% of total fatty acids, respectively. Densipolic acid and 16:2-9c,12t

Figure 1. An unrooted phylogenetic analysis based on CpFAH and related enzymes. The GenBank accession numbers of the sequences are indicated in parentheses: CpFAH, C. purpurea 12-hydroxylase (EU661785; this report); CpDes12, C. purpurea Δ12 desaturase (EF536897); CpDesX, C. purpurea novel desaturase (EF536898); AtFAD2, Arabidopsis Δ12 desaturase (P46313); RcFAH, castor Δ12 desaturase (AKS59093); RcFAH, castor 12-hydroxylase (AAC49010); LfFAH, L. fendleri 12-hydroxylase (AAC32755); FmDes12, Fusarium moniliforme Δ12 desaturase (DQ272515); AnDes12, A. nidulans Δ12 desaturase (XP_658641).
were minor products, in combination representing less than 1.5% of the total fatty acids. The 12-hydroxylation activity of CpFAH was almost 7 times the \( \Delta^{12} \) desaturation activity on oleic acid, indicating hydroxylation of oleic acid is the major activity of this enzyme in yeast.

**Expression of CpFAH in Arabidopsis**

To assess the capability of the fungal hydroxylase to synthesize hydroxyl fatty acids in planta, \( \text{CpFAH} \) under the control of the seed-specific napin promoter was transformed into wild-type and \( \text{fad2} / \text{fae1} \) mutant Arabidopsis plants, along with the \( \text{DsRed2} \) reporter gene. The Arabidopsis mutant line (\( \text{fad2} / \text{fae1} \)), which carries mutations in both the \( \text{FAD2} \) and \( \text{FAE1} \) genes, produces a high level of substrate fatty acid (oleate) that can be used for the synthesis of ricinoleic acid (Smith et al., 2003). Use of the \( \text{DsRed2} \) reporter gene permits the easy identification of transgenic seeds by examination under green illumination using a red filter (Lu et al., 2006). A floral-dip transformation approach (Clough and Bent, 1998) was used to obtain 11 transgenic plants from each of the two genotypes used in this study. Typical chromatograms from GC analysis of FAME derivatives of transgenic seeds are shown in Figure 4. The most abundant fatty acid in untransformed wild-type Arabidopsis seeds was 18:2-9c,12c, followed by 20:1-11c, 18:3-9c,12c,15c, and 18:1-9c. Minor components included 16:0 and 18:0. Expression of CpFAH in wild-type Arabidopsis resulted in the production of three hydroxy fatty acids; among these, ricinoleic acid, which is produced via the direct hydroxylation of oleic acid by CpFAH, was the most abundant, followed by densipolic acid, derived from the \( \omega^{3} \) desaturation of ricinoleic acid, and lesquerolic acid, a 20C hydroxyl fatty acid derived from the elongation of ricinoleic acid (Fig. 4A; Table I). However, unlike yeast, transformed wild-type Arabidopsis did not produce a detectable level of 12-OH-16:1-9c, which may have been due to the low availability of the 16:1-9c substrate.

The untransformed mutant Arabidopsis, because it lacks activities of both the \( \Delta^{12} \) desaturase (\( \text{FAD2} \)) and a condensing enzyme or elongase (\( \text{FAE1} \)), accumulated substantial amounts of oleic acid. Expression of \( \text{CpFAH} \) in this line resulted in the production of two hydroxyl fatty acids (ricinoleic acid and densipolic acid) and one \( \Delta^{12} \) desaturated fatty acid (linoleic acid) with ricinoleic acid predominating (Fig. 4B). Although oleic acid was still the most abundant fatty acid, its

![Figure 3](image3.png)  
**Figure 3.** Mass spectra of FAME-TMS derivatives of hydroxyl fatty acid peaks from Figure 2. The identity of the peaks were confirmed as 12-OH-18:1-9c (A), 12-OH-18:2-9c,15c (B), and 12-OH-16:1-9c (C).

![Figure 4](image4.png)  
**Figure 4.** GC analysis of FAMES prepared from mature seeds of Arabidopsis. A, Wild-type (1) untransformed, and (2) transformed with \( \text{CpFAH} \); B, \( \text{fad2} / \text{fae1} \) double mutant untransformed (1) and transformed with \( \text{CpFAH} \) (2).
than were seen with the expression of plant hydroxyl fatty acids (Table I), with the highest level in a single seed reaching 29.1%. These are considerably higher levels than were seen in yeast and plant expression of CpFAH in the transformed mutant Arabidopsis seeds. The amount of hydroxyl fatty acids produced in transformed wild-type Arabidopsis ranged from 15.9% to 22.0%. Heterologous expression of CpFAH in the transgenic plants (Table I). The production of the untransformed control to approximately 45% in the transgenic lines is depicted in Figure 5. The total hydroxyl fatty acids in transformed wild-type Arabidopsis seeds (fad2/fae1) transgenics have rarely exceeded 20% of the total fatty acids in seeds in these lines (Smith et al., 2003).

The values are derived from GC analysis of FAME-TMS derivatives (mean ± SE).

level was dramatically reduced from more than 80% in the untransformed control to approximately 45% in the transgenic plants (Table I). The production of linoleic acid in transformed mutant Arabidopsis seeds confirmed that CpFAH exhibited Δ12 desaturase activity in plants, as was also observed in yeast.

The amount of hydroxyl fatty acids produced in transgenic lines is depicted in Figure 5. The total hydroxyl fatty acids in transformed wild-type Arabidopsis ranged from 15.9% to 22.0%. Heterologous expression of CpFAH in the fad2/fae1 genotype led to the accumulation of 17.3% to 25% of hydroxyl fatty acids (Table I), with the highest level in a single seed reaching 29.1%. These are considerably higher levels than were seen with the expression of plant hydroxyl fatty acids in either wild-type or mutant Arabidopsis plants (Broun and Somerville, 1997; Smith et al., 2003). A large number of Arabidopsis plants carrying either castor or Lesquerella hydroxylases in different genetic backgrounds (wild type, fad3, fad2/fae1, fad3/ fad1) have been produced; however, hydroxyl fatty acids have rarely exceeded 20% of the total fatty acids in seeds in these lines (Smith et al., 2003).

**DISCUSSION**

Although it has long been known that the fungus C. purpurea produces substantial amounts of ricinoleic acid (Morris and Hall, 1966), the mechanism for the biosynthesis of this fatty acid has yet to be elucidated. It has been believed for almost 40 years that its biosynthesis in C. purpurea involves a hydration process that introduced a water molecule into linoleic acid, giving rise to ricinoleic acid (Morris et al., 1966; Morris, 1970). However, a recent study using deuterium NMR rebuffed this hypothesis and showed that the synthesis of ricinoleic acid in C. purpurea follows an oxygen-dependent hydroxylation process using oleic acid as the substrate (Billault et al., 2004), although the exact nature of the hydroxylase enzyme was not determined. Two possibilities existed; the hydroxylation of oleic acid could be catalyzed by a desaturase-like hydroxylase as occurs in plants or by a cytochrome P450-like hydroxylase as is seen in some fungal species. For example, the fungus Aspergillus nidulans uses a family of oxygenated long-chain fatty acids called psi factors (precocious sexual inducers), such as psiβ (8-hydroxy-18:2,9,12) and psiCa (5,8-dihydroxy-18:2,9,12), to modulate sexual and asexual spor development. Both these hydroxyl fatty acids are synthesized by cytochrome...
P450-like fatty acid oxygenases (Tsitsigiannis et al., 2004, 2005). In this study, we focused on the possibility that the biosynthesis of ricinoleic acid in *C. purpurea* resembles that in plants, where the oleate hydroxylase has high sequence similarity to oleate desaturases (van de Loo et al., 1995). We designed two degenerate primers targeting fungal oleate desaturases and used RNA isolated from *C. purpurea* sclerotia as the template for RT-PCR. By this method we were able to identify a cDNA (*CpFAH*) from *C. purpurea* that encodes an enzyme that preferentially introduces a hydroxyl group into position 12 of oleic acid. *CpFAH* represents the first 12-oleate hydroxylase of nonplant origin. Compared to its plant counterparts, *CpFAH* appears to have less strict substrate specificity. As well as showing strong hydroxylase activity on oleate and palmitoleate, *CpFAH* also catalyzes Δ\(^{12}\) desaturation of oleate and palmitoleate and, to a limited extent, is able to desaturate linoleate and ricinoleate at the \(\omega_3\) position.

Sequence analysis of *CpFAH* indicates it shares approximately 80% amino acid identity with both *CpDes12* (a Δ\(^{12}\) desaturase) and *CpDesX* (a novel desaturase) from *C. purpurea* (Meesapyodsuk et al., 2007), but only approximately 40% amino acid identity with its plant counterparts—castor bean and *Lesquerella* hydroxylases. Phylogenetic analysis suggests that the *CpFAH* gene of *C. purpurea* might have evolved independently from its own prototype Δ\(^{12}\) desaturase, rather than from an ancient common ancestral 12-hydroxylase or from a Δ\(^{12}\) desaturase shared by plants and fungi. Many common features of fatty acid desaturases and fatty acid hydroxylases, such as their strikingly similar hydropathy profiles that predict similar membrane topology, and conserved His-rich motifs thought to be responsible for diiron binding at the catalytic center, point to a common evolutionary relationship for these two types of enzymes (Shanklin and Cahoon, 1998; Sperling et al., 2003). Indeed, site-directed mutagenesis experiments have shown that as few as four amino acid substitutions can convert an Arabidopsis oleate desaturase to an oleate hydroxylase (Broun et al., 1998b; Broadwater et al., 2002). In *C. purpurea*, *CpDes12*, *CpDesX*, and *CpFAH* share striking similarity to each other in primary sequence but possess distinct functions. *CpDes12* is a typical Δ\(^{12}\) desaturase active on monounsaturated fatty acids such as oleic and palmitoleic acids. *CpDesX* is a desaturase with Δ\(^{12}\), Δ\(^{15}\), and \(\omega_3\) types of desaturation activities.

**Figure 5.** Hydroxyl fatty acid content of mature seeds from individual transgenic Arabidopsis plants expressing *CpFAH*. A, Wild type. B, *fad2/fae1* double mutant. Transgenic seeds were screened using the DsRed2 fluorescent marker. Values are measurements of five T\(_2\) seeds.
although \( \omega^3 \) activity on linoleic acid predominates. The primary activity of CpFAH is that of an oleic acid hydroxylase, although it is also capable of introducing \( \Delta^{12} \) double bonds to oleic and palmitoleic acids. The high sequence similarity of these enzymes coupled with their distinct functions provides a unique opportunity to investigate the influence of individual amino acids on substrate specificity, regioselectivity, and function.

Metabolic engineering of plants to produce unusual fatty acids for industrial applications is the ultimate goal of studies on the biosynthesis and bioassembly of unusual fatty acids. Many genes encoding enzymes involved in the synthesis of these fatty acids have recently been identified from native plant species. However, to date the production of unusual fatty acids through the introduction of these genes into oilseed crops has met with only partial success. Although proof of concept has been achieved by the production of many unusual fatty acids in transgenic crops, commercially viable levels have generally yet to be attained (Cahoon et al., 2007; Napier, 2007). The use of genetically engineered plants to produce ricinoleic acid has drawn a particularly high level of interest due to its specialized industrial use. Three oleate hydroxylase genes from castor and Lesquerella have been transformed into Arabidopsis, tobacco (Nicotiana tabacum), and Brassica napus; however, the hydroxyl fatty acid content in transgenic seeds has rarely exceeded 20% of the total fatty acids (van de Loo et al., 1995; Broun and Somerville, 1997; Broun et al., 1998a; Smith et al., 2003). Although it is not yet known why the level of hydroxyl fatty acid content in transgenic plants is so much lower than that in native species, it has been hypothesized that contributing factors may include the inefficient removal of newly synthesized unusual fatty acids from membrane phospholipids (Thomäus et al., 2001; Cahoon et al., 2006) and the inefficient transfer of newly synthesized unusual fatty acids to storage lipids (Cahoon et al., 2007; Napier, 2007).

Expression of the C. purpurea CpFAH in transgenic plants resulted in the accumulation of substantial amounts of hydroxyl fatty acids and a small amount of linoleic acid in the double mutant Arabidopsis line. The latter is derived from the minor \( \Delta^{12} \) desaturase activity of the transgene on oleic acid. The hydroxyl fatty acids in the transgenic mutant Arabidopsis reached 25% of the total fatty acids in bulked seeds, with a maximum level of 29.1% in a single seed of a T3 transgenic plant. Thus, it appears that selection of genes from diverse sources, especially from nonplant sources, can make a positive contribution to transgenic production of unusual fatty acids. The reason for the better performance of the fungal hydroxylase is currently unknown, but might be simply that CpFAH itself has higher hydroxylase activity. Furthermore, since the fungal oleate hydroxylase has less similarity than plant counterparts to endogenous homologous desaturase genes, CpFAH might be subject to less stringent regulatory control by the host plant. Regardless of the cause, the higher hydroxylase activity of CpFAH in both yeast and plants makes this gene a good candidate for use in metabolic engineering of hydroxyl fatty acids in oilseed crops.

**MATERIALS AND METHODS**

**Organisms and Culture Conditions**

To produce sclerotium-forming mycelia, *Claviceps purpurea* was routinely maintained at 25°C for 14 d in medium C or for 20 to 30 d in amino acid medium (Mantle and Nisbet, 1976; Meesapyodsuk et al., 2007).

**Cloning of CpFAH cDNA from C. purpurea**

Total RNA was extracted from sclerotium-forming mycelia of *C. purpurea* using TRIzol reagent (Invitrogen), and 5 μg was used to synthesize first-strand cDNA using the SuperScript III first-strand synthesis system (Invitrogen). Two microliters of the first-strand cDNA was used as a template for PCR amplification with two degenerate oligonucleotide primers (DM34 and DM36) that were designed based on conserved amino acid sequences of \( \Delta^2 \) and bifunctional \( \Delta^{12}/\Delta^9 \) desaturases from fungi (Damude et al., 2006; Meesapyodsuk et al., 2007). The detailed sequences of all primers used in this study are shown in Table II. The forward primer (DM34) corresponds to the first conserved His box and has the amino acid sequence AHECGHG/QAF, while the reverse primer (DM36) lies between His boxes 2 and 3 and corresponds to the amino acid sequence WV(N/H)HWLYAFTY. Amplified products having the expected size of approximately 600 bp were gel purified, then cloned into the pCR4-TOPO vector (Invitrogen) and sequenced.

The 5′ and 3′ ends of the CpFAH cDNA were obtained using the Marathon cDNA amplification kit (BD Biosciences, CLONTECH) following the manufacturer’s recommendations. Primers DM58 and DM59 were used to obtain the 5′ and 3′ ends, respectively. The full-length sequence, including both untranslated and coding regions, was then amplified using Phs50 DNA polymerase (Invitrogen) with specific primers DM61 and DM62. The resulting PCR products were cloned into the pCR4-TOPO vector, producing construct pDM15.

**Phylogenetic Analysis**

Homologous sequences of CpFAH from fungi and plants were identified by BLASTP searches using CpFAH as a query against public databases at the National Center for Biotechnology Information. Six highly homologous sequences of fungal \( \Delta^2 \) and bifunctional \( \Delta^{12}/\Delta^9 \) desaturases from *Aspergillus nidulans* and *Fusarium oxysporum*, along with four well-known \( \Delta^2 \) desaturases and hydroxylases from the plants Arabidopsis (*Arabidopsis thaliana*), castor (*Ricinus communis*), and Lesquerella (*Lesquerella fendleri*) were chosen for construction of the phylogenetic tree. Multiple alignment of amino acid sequences was performed using ClustalW multiple alignment software (DNAStar) with default parameters. An unrooted phylogenetic tree was generated from the alignment and displayed using TreeView (Page, 1996).

**Functional Analysis of CpFAH in Yeast**

The coding region of the CpFAH cDNA was amplified from pDM15 using Phs50 DNA polymerase with primers DM36 and DM64. After a brief Taq DNA polymerase (Invitrogen) treatment, the amplified fragment was cloned directly into the pYES21-TOPO expression vector (Invitrogen). The resulting plasmid construct, pDM16, was introduced into yeast *S. cerevisiae* strain INVSc1 (MATa his3A1 leu2 trp1-289 ura3-52 MATa his3A1 leu2 trp1-289 ura3-52; Invitrogen) using the S.C. EasyComp transformation kit (Invitrogen). For expression studies, yeast transformed with either pDM16 or the empty vector pYES21 were grown at 28°C for 2 d in synthetic dropout medium containing 0.17% (w/v) yeast nitrogen base, 0.5% ammonium sulfate, 2% (w/v) dextrose, and 0.06% (w/v) dropout supplement lacking uracil. Following two washes with sterile water, the expression of the transgene in this study were shown to be reached 25% of the total fatty acids in bulked seeds, with a maximum level of 29.1% in a single seed of a T3 transgenic plant. Thus, it appears that selection of genes from diverse sources, especially from nonplant sources, can make a positive contribution to transgenic production of unusual fatty acids. The reason for the better performance of the fungal hydroxylase is currently unknown, but might be simply that CpFAH itself has higher hydroxylase activity. Furthermore, since the fungal oleate hydroxylase has less similarity than plant counterparts to endogenous homologous desaturase genes, CpFAH might be subject to less stringent regulatory control by the host plant. Regardless of the cause, the higher hydroxylase activity of CpFAH in both yeast and plants makes this gene a good candidate for use in metabolic engineering of hydroxyl fatty acids in oilseed crops.
Plant Vector Construction

The expression vector pDM22 with CpfAH from C. purpurea and DsRed2 from Discocoma sp. encoding a red fluorescent protein, each under control of a seed-specific napin promoter, was constructed as described previously (Wu et al., 2005). DsRed2 was PCR amplified from plasmid pDSRed2-C1 (Tzfira et al., 2003) with primers DM81 and DM62, and CpfAH was PCR amplified from pDM16 with primers DM68 and DM69. Primers DM81 and DM82 introduce BamHI restriction sites, while primers DM68 and DM69 introduce XhoI restriction sites. Amplified fragments were first cloned into the pCR4-TOPO vector and sequenced. The fragments were then released by digestion with the appropriate restriction enzymes and sequentially subcloned into pUC19 between napin promoters and octopine synthase terminators. The two-gene cassette was removed from pUC19 by EcoRI digestion and inserted into the EcoRI site of a plant-expression vector, producing construct pDM22.

Production of Transgenic Arabidopsis

The construct pDM22 was introduced into Agrobacterium tumefaciens strain GV3101 (pMP90) by electroporation. Arabidopsis wild-type (Columbia ectoype) and fad2/fad1 mutant lines (Smith et al., 2003) were transformed using the floral dip method (Clough and Bent, 1998). Selection of transformants was based on either kanamycin resistance (T1 seeds) or DsRed2 expression (T2 seeds). Plants were grown in a growth chamber at 22°C under a 16-h-light (120 μE m⁻² s⁻¹)/8-h-dark photoperiod.

Fatty Acid Analysis

Yeast fatty acids were extracted and methylated as previously described (Meesapyodsuk et al., 2007). Arabidopsis transgenic seeds identified by DsRed2 expression were analyzed by GC or GC-MS of FAMEs and/or TMS derivatives. Seeds were crushed with glass rod and FAMEs were derived from napin (120 μg) with 0.25-microliter samples of total FAMEs and/or TMS derivatives. Seeds were analyzed on a gas chromatograph equipped with a DB-23 column (30 m x 0.25 mm) with 0.25-μl oven temperature maintained at 160°C for 1 min, then increased to 240°C at a rate of 4°C/min. For MS, the mass selective detector was run under standard electron impact conditions (70 eV), scanning an effective m/z range of 40 to 700 at 2.26 scans/s. Fatty acids were identified by comparison of retention times with authentic fatty acid standards, whereas hydroxylated derivatives of fatty acids were identified by MS on the basis of their fragmentation patterns as previously reported (van de Loo et al., 1995; Broun and Somerville, 1997). The nucleotide sequence reported in this article has been submitted to GenBank with accession number EU661785.

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