Divergent forms of the plant Δ^{12}-oleic-acid desaturase (FAD2) have previously been shown to catalyze the formation of acetylenic bonds, epoxy groups, and conjugated Δ^{11},Δ^{13}-double bonds by modification of an existing Δ^{12}-double bond in C_{18} fatty acids. Here, we report a class of FAD2-related enzymes that modifies a Δ^{9}-double bond to produce the conjugated trans-Δ^{9},trans-Δ^{12}-double bonds found in calendic acid (18:3Δ^{9,12,15}(cis,cis,trans)), the major component of the seed oil of Calendula officinalis. Using an expressed sequence tag approach, cDNAs for two closely related FAD2-like enzymes, designated CoFADX-1 and CoFADX-2, were identified from a C. officinalis developing seed cDNA library. The deduced amino acid sequences of these polypeptides share 40–50% identity with those of other FAD2 and FAD2-related enzymes. Expression of either CoFADX-1 or CoFADX-2 in somatic soybean embryos resulted in the production of calendic acid. In embryos expressing CoFADX-2, calendic acid accumulated to as high as 22% (w/w) of the total fatty acids. In addition, expression of CoFADX-1 and CoFADX-2 in Saccharomyces cerevisiae was accompanied by calendic acid accumulation when induced cells were supplied exogenous linoleic acid (18:2Δ^{cis,cis,12cis}). These results are thus consistent with a route of calendic acid synthesis involving modification of the Δ^{9}-double bond of linoleic acid. Regiospecificity for Δ^{9}-double bonds is unprecedented among FAD2-related enzymes and further expands the functional diversity found in this family of enzymes.

The common polysaturated fatty acids of plant seed oils contain cis-double bonds that are separated by a methylene group. The primary examples of such fatty acids are linoleic acid (18:2Δ^{cis,12cis}) and α-linolenic acid (18:3Δ^{cis,cis,12cis}). In contrast, the seed oils of a number of plant species contain polysaturated fatty acids with conjugated (or non-methylene-interrupted) double bonds (1). Examples of these unusual fatty acids include α-eleostearic acid (18:3Δ^{cis,cis,12 cis,15cis}), α-parinaric acid (18:4Δ^{cis,cis,11trans,13trans,15cis}), punicic acid (18:3Δ^{cis,cis,11trans,13cis}), and calendic acid (18:3Δ^{cis,cis,11trans,13cis,15cis}) (1). Seed oils that contain fatty acids with conjugated double bonds display high rates of oxidation compared with oils that contain unsaturated fatty acids with methylene-interrupted double bonds (2). Because of this property, seed oils such as tung oil that are enriched in fatty acids with conjugated double bonds are used commercially as drying agents in paints and varnishes (3).

We have recently demonstrated that the conjugated trans-Δ^{11}, and trans-Δ^{13}-double bonds of α-eleostearic and α-parinaric acids in seeds of Momordica charantia and Impatiens balsamina, respectively, are synthesized by divergent forms of the Δ^{12}-oleic-acid desaturase (FAD2; oleate desaturase, EC 1.3.1.35), which we have termed “conjugases” (4). These enzymes catalyze the conversion of an existing cis-Δ^{12}-double bond into conjugated trans-Δ^{11}, and trans-Δ^{13}-double bonds (4, 5). This activity contrasts with that of the typical FAD2 desaturase of plants, which introduces a cis-Δ^{12}-double bond into oleic acid. In M. charantia seeds, α-eleostearic acid is formed by modification of the cis-Δ^{12}-double bond of linoleic acid by a FAD2 conjugase (4). Similarly, the synthesis of α-parinaric acid in I. balsamina seeds arises from the conjugase-catalyzed modification of the cis-Δ^{12}-double bond of α-linolenic acid (4). These reactions use fatty acids bound to phosphatidylethanolamine as substrates (5), as has been shown for other FAD2-type enzymes (6). In addition, based on the mechanism proposed for conjugated double bond synthesis in red algae (7), the production of α-eleostearic acid and α-parinaric acids probably involves removal of a hydrogen atom from the C-11 and C-14 methylene groups that flank the cis-Δ^{12}-double bond of linoleic and α-linolenic acids.

Calendic acid, the primary fatty acid of Calendula officinalis seeds (8–10), is a conjugated trienoic fatty acid, like α-eleostearic acid, but contains conjugated trans-Δ^{9}, trans-Δ^{12}, and cis-Δ^{12}-double bonds. This fatty acid contains 50–60% (w/w) of the seed oil of C. officinalis, but is absent from leaves of this plant (8). In common with α-eleostearic acid synthesis, linoleic acid has been shown to be the biosynthetic precursor of calendic acid (11, 12). Unlike α-eleostearic acid synthesis, however, the conjugated trans-Δ^{9}- and trans-Δ^{12}-double bonds of calendic acid arise from modification of the cis-Δ^{9}-double bond of linoleic acid (11, 12). Based on our previous studies (4) and the proposed mechanism of conjugases (7), it seemed likely that the conjugated trans-Δ^{9}- and trans-Δ^{12}-double bonds of calendic acid are formed by a fatty acid desaturase-like enzyme. The involvement of a FAD2-related enzyme in the modification of a...
cis-Δ9-double bond, however, has not been previously demonstrated.

In this report, we have undertaken a genomics-based approach to characterize the biosynthetic origin of the conjugated double bonds of calendic acid. By sequencing of random cDNAs derived from developing C. officinalis seeds, we have identified cDNAs for two closely related variant forms of FAD2. Expression of either cDNA in somatic soybean embryos results in the accumulation of calendic acid. These findings thus demonstrate that FAD2-type enzymes can catalyze not only the modification of the Δ12- position, but also the Δ9- position of fatty acid substrates. In addition, we show that calendic acid accumulation in somatic soybean embryos is not accompanied by large increases in oleic acid content, which is in contrast to the phenotype generally observed with the expression of other divergent FAD2 enzymes in transgenic plants (4, 13, 14).

EXPERIMENTAL PROCEDURES

cDNA Library Construction—Total RNA was isolated from developing seeds of C. officinalis variety Dwarf Gem (Burpee) plants using the method described by Jones et al. (15). Poly(A)+ RNA was enriched from the total RNA and used for cDNA library construction as described previously (4). The resulting library consisted of cDNA inserts cloned directionally (5'-3') in the EcoRI and XhoI sites of pBluescript II SK (+) to maintain in Escherichia coli DH10B cells (Life Technologies, Inc.). Bacterial cells harboring the libraries in plasmid form were stored as glycerol stocks at −80 °C until used for expressed sequence tag (EST)3 analysis.

Generation of ESTs and Identification of Divergent FAD2 cDNAs—Plasmids for EST analysis were prepared from randomly picked colonies from the C. officinalis cDNA library using the QIAGEN REAL Prep 96 system according to the manufacturer’s protocol. Nucleotide sequence was obtained from the 5'-ends of cDNAs in pBluescript II SK(+) using the M13 reverse priming site and dye terminator cycle sequencing with an ABI 377 DNA fluorescence sequencer. Partial nucleotide sequences were obtained for 3036 random cDNAs from the C. officinalis developing seed library using this methodology. Putative identities were assigned to these cDNAs by comparison of their partial sequences with translated sequences in the public data bases using the NCBI BLASTX program (16).

From this analysis of the C. officinalis developing seed library, full-length cDNAs for two closely related divergent forms of FAD2 were identified. The polypeptides encoded by these cDNAs were designated CoFADX-1 and CoFADX-2. Nucleotide sequences were determined from both strands of the CoFADX-1 and CoFADX-2 cDNAs in pBluescript II SK(+) by dye terminator sequencing using the instrumentation described above.

Expression of CoFADX-1 and CoFADX-2 cDNAs in Somatic Soybean Embryos—The vector pKS67 was used for expression of cDNAs for CoFADX-1 and CoFADX-2 in soybean (Glycine max) somatic embryos. This vector contains a unique NotI site for cloning of transgenes that is flanked by the seed-specific promoter of the gene for the α-subunit of β-conglycinin (17) and phaseolin termination sequence (18). Bacterial selection with this vector is conferred by a hygromycin B phosphotransferase gene (19) under the control of the T7 RNA polymerase promoter, and plant selection is conferred by a second hygromycin B phosphotransferase gene under the control of the cauliflower mosaic virus 35S promoter.

The coding sequences for CoFADX-1 and CoFADX-2 were amplified by PCR using Phu polymerase (Stratagene) to generate flanking NotI sites for subcloning into the pKS67 expression vector. Full-length cDNAs for CoFADX-1 and CoFADX-2 were used as templates for PCRs. For amplification of CoFADX-1, the following oligonucleotide primer combination was used: 5'-tggcgccgctACCTCAGCTGATCCCCGAGC-3' (sense) and 5'-ttggcgccgCTACCACTGATCATGATGGCCAC-3' (antisense). The CoFADX-2 cDNA was amplified using the following primer combination: 5'-tggcgccgTCATGATGGCCAAAGAGACTGATGCGAGCA-3' (sense) and 5'-ttggcgccgTGGAGACTCTTTCACCTGTGGATCTGGGGGC-3' (antisense). Note that the sequences shown in lowercase letters contain an added NotI site along with additional bases to facilitate restriction enzyme digestion. The resulting PCR products were subcloned into the intermediate vector pCR-Script Amp SK (+) (Stratagene) according to the manufacturer’s protocol. The amplified coding sequence for CoFADX-1 or CoFADX-2 was then released with NotI digestion and subcloned into the corresponding site of the pKS67 expression vector.

Gene fusion of the CoFADX-1 and CoFADX-2 cDNAs with the β-conglycinin promoter and phaseolin termination sequences in vector pKS67 were introduced into soybean embryos of cultivar A2872 or Jack using the particle bombardment method of transformation (4, 20). Selection and propagation of the transgenic somatic soybean embryos have been described previously (4, 20). Expression of CoFADX-1 or CoFADX-2 was confirmed by PCR amplification using sequence-specific primers and first-strand cDNA prepared from total RNA isolated from the transgenic somatic soybean embryos.

Fatty Acid Analysis of Transgenic Soybean Embryos—Fatty acid methyl esters were prepared from transgenic soybean embryos by transesterification in 1% (w/v) sodium methoxide in methanol. Single soybean embryos were homogenized with a glass stirring rod in 0.5 ml of the sodium methoxide solution and incubated at room temperature for 20 min. At the end of this period, 0.5 ml of 1 M sodium chloride was added, and fatty acid methyl esters were extracted with 0.5 ml of hexane. Fatty acid methyl esters were separated and quantified using a Hewlett-Packard 5890 gas chromatograph fitted with an Omegawax column (30 m × 0.22 mm, inner diameter; Supelco Inc.). The oven temperature was programmed from 150 °C (initial temperature) to 220 °C (final temperature) at a rate of 20 °C/min, and carrier gas was supplied by a Whatman hydrogen generator. Fatty acid methyl esters were also analyzed by GC-MS using a Hewlett-Packard 6890 gas chromatograph interfaced with a Hewlett-Packard 5973 mass selective detector. Samples were separated with an INNOWax column (30 m × 0.25 mm (inner diameter); Hewlett-Packard Co.) or an HP-5 column (30 m × 0.25 mm (inner diameter); Hewlett-Packard Co.). The oven temperature was programmed from 150 °C (initial temperature) to 275 °C at a rate of 2.5 °C/min and then to 230 °C at a rate of 5 °C/min. The structures of fatty acid methyl esters with conjugated double bonds were also characterized by GC-MS following Diels-Alder derivatization by reaction with 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (Aldrich) (21). For these studies, fatty acid methyl esters from transgenic soybean embryos were reacted for 10 s on ice with 0.5 ml of 5 mM MTAD in dichloromethane. The reaction was stopped by the addition of 50 μl of 2.4-hexadiene (Aldrich). The derivatized samples were then dried under nitrogen and resuspended in heptane for GC-MS analysis. MTAD adducts were resolved using either a DB-1 column (15 m × 0.25 mm (inner diameter); J&W Scientific) or an HP-5 column (30 m × 0.25 mm (inner diameter); Hewlett-Packard Co.). The oven temperature was programmed from 150 °C (initial temperature) to 230 °C at a rate of 20 °C/min. For identification of calendic acid, the mass spectra of Diels-Alder derivatives prepared from transgenic soybean embryos were compared with those of calendic acid adducts generated from fatty acid methyl esters of C. officinalis seeds.

Expression of CoFADX-1 and CoFADX-2 in Saccharomyces cerevisiae—The activities of CoFADX-1 and CoFADX-2 were characterized by expression in the corresponding full-length cDNAs in the yeast S. cerevisiae. The GAL1 promoter in the vector pESC-URA (Stratagene) was used to express the corresponding cDNAs in the yeast S. cerevisiae INVSc1 cells (Invitrogen) by lithium acetate-mediated transformation (22). Transformed cells were selected for their ability to grow on medium lacking uracil. Individual colonies of transformed yeasts were then grown for 2 days at 30 °C in medium lacking uracil (0.17% (w/v) yeast nitrogen base without amino acids (Difco), 0.5% (w/v) ammonium sulfate, and 0.18% (w/v) SC-URA (Bio 101, Inc.)) supplemented with glycerol and glucose to final concentrations of 5% (v/v) and 0.5% (w/v), respectively. Cells were then washed twice in the growth medium described above with galactose at a final concentration of 2% (w/v) as the carbon source. The washed cells were then diluted to A500 = 0.2 in the galactose growth medium that also contained Tergitol type NP-40 (Sigma) at a concentration of 0.2% (w/v). Aliquots of these cells were grown in a volume of 3 ml without exogenous fatty acids or with the addition of oleic acid (18:1Δ9cis, 0.15%), linoleic acid (18:2Δ9,12cis, 1%), or linolenic acid (18:3Δ9,12,15cis, 1%) at a final concentration of 0.7 μM. Experiments were also conducted with both linoleic and linolenic acids added to the medium, each at a concentration of 0.35 mM. Galactose-induced cultures were maintained at 16 °C with shaking (350 rpm). Cells were harvested by centrifugation when cultures reached densities of A500 = 3–4. Cell pellets from the 3-ml cultures were washed with water and dried under vacuum. The pellets were then resuspended in 0.4 ml of 1% (w/v) sodium methoxide in methanol and incubated at 60 °C for 0.5 h with gentle vortexing. The pellets were then applied to a column of the sodium methoxide solution and incubated at room temperature for 20 min. At the end of this period, 0.5 ml of 1 M sodium chloride was added, and fatty acid methyl esters were extracted with 0.5 ml of hexane. Fatty acid methyl esters were separated and quantified using a Hewlett-Packard 5890 gas chromatograph interfaced with a Hewlett-Packard 5973 mass selective detector. Samples were separated with an INNOWax column (30 m × 0.25 mm (inner diameter); Hewlett-Packard Co.) or an HP-5 column (30 m × 0.25 mm (inner diameter); Hewlett-Packard Co.). The oven temperature was programmed from 150 °C (initial temperature) to 275 °C at a rate of 2.5 °C/min. For identification of calendic acid, the mass spectra of Diels-Alder derivatives prepared from transgenic soybean embryos were compared with those of calendic acid adducts generated from fatty acid methyl esters of C. officinalis seeds.

3 The abbreviations used are: EST, expressed sequence tag; PCR, polymerase chain reaction; GC-MS, gas chromatography-mass spectrometry; MTAD, 4-methyl-1,2,4-triazoline-3,5-dione.
room temperature for 20 min. Fatty acid methyl esters resulting from this direct transesterification of cell pellets were extracted and analyzed by GC and GC-MS as described above for analysis of somatic soybean embryos. Fatty acid methyl esters were also reacted with MTAD, and the resulting Diels-Alder adducts were analyzed by GC-MS as described above.

Northern Blot Analysis—Total RNA was extracted from leaves and developing seeds of *C*. *officinalis* using Trizol (Life Technologies, Inc.) according to the manufacturer’s protocol. Total RNA (20 μg) from each tissue and RNA standards were electrophoresed on a 1% (w/v) agarose gel containing formaldehyde. Following electrophoresis, RNA was transferred from the gel to Bright Star-Plus nylon membrane (Ambion Inc.). The RNA was fixed to the membrane by UV cross-linking. The membrane was rinsed with 2× SSC and then hybridized with 32P-labeled probes for 18 h at 42 °C in NorthernMax hybridization buffer (Ambion Inc.).

Probes were prepared from cDNAs for CoFADX-1 or CoFADX-2 and labeled using random hexamer priming (17). Following incubation with probes, blots were pre-washed for 15 min at room temperature with 0.2× SSC and 0.1% SDS. Radioactivity on filters was detected by phosphorimaging. Message sizes were estimated based on mobility relative to a 0.24–9.5 kilobase RNA ladder (Life Technologies, Inc.).

Given the high degree of identity between the open reading frames of the CoFADX-1 and CoFADX-2 cDNAs, probes were prepared primarily from the 3′-untranslated regions of these cDNAs to more specifically distinguish between the expression patterns of the corresponding genes. The probes used for Northern analysis were generated by PCR amplification using *Pfu* polymerase, and cDNAs for CoFADX-1 and CoFADX-2 were used as templates. PCR products were purified by agarose gel electrophoresis prior to use in labeling reactions. For amplification of the CoFADX-1-specific probe (292 base pairs), the following oligonucleotides were used: 5′-GATTGGAAGTTTCAATAATC-3′ (sense) and 5′-GATAACGCCTTTATTATACTG-3′ (antisense). For amplification of the CoFADX-2-specific probe (149 base pairs), the following oligonucleotides were used: 5′-CTTCCTGGAAGAGGCTGAGT-3′ (sense) and 5′-GATTTGAAGTTTCAAATAATC-3′ (antisense).

RESULTS

Identification of Divergent FAD2 cDNAs in *C*. *officinalis* Seeds—An EST approach was used to determine the biosynthetic origin of calendic acid in *C*. *officinalis* seeds. DNA sequences were obtained from the 5′-ends of >3000 randomly selected cDNAs from a *C*. *officinalis* developing seed library. From this pool of ESTs, 12 cDNAs that encode FAD2-related polypeptides were identified by BLAST homology. Based on sequence comparisons, five of these cDNAs corresponded to plant FAD2 polypeptides that were more closely related to the typical plant FAD2 that is associated with the cis-Δ12-desaturation of oleic acid. The remaining seven cDNAs were found to encode two closely related polypeptides that were designated CoFADX-1 and CoFADX-2. Of these cDNAs, six encoded CoFADX-1, and one encoded CoFADX-2. The longest full-length cDNAs corresponding to CoFADX-1 and CoFADX-2 contained 1457 and 1295 base pairs, respectively. The amino acid sequences of CoFADX-1 and CoFADX-2 deduced from full-length cDNAs share 94% identity (Fig. 1). These polypeptides, however, share >51% identity with all reported FAD2 and FAD2-like enzymes, including hydroxylases (14, 23), epoxygenases (24), acyltransferases (24), and Δ3-specific conjugases (4) (Figs. 1 and 2). Using Northern blot analysis, expression of genes for CoFADX-1 and CoFADX-2 was detected in developing seeds, but was not detected in leaves of *C*. *officinalis* (Fig. 3). This expression profile is consistent with the seed-specific occurrence of calendic acid in *C*. *officinalis* (8, 28).

Functional Characterization of Divergent *C*. *officinalis* FAD2 Enzymes in Transgenic Plants—FAD2-related polypeptides are microsomal enzymes that are typically recalitrant to in vitro assay in solubilized membrane extracts (6). As an alternative method of functional characterization, CoFADX-1 and Co-FAD2 were expressed in somatic soybean embryos to examine their effect on fatty acid content. Like seeds, somatic soybean embryos are rich in triacylglycerols, and the fatty acid composition of transgenic somatic embryos is completely predictive of the fatty acid composition of seeds obtained from regenerated plants (25). In these experiments, expression of cDNAs for CoFADX-1 and CoFADX-2 was placed under the
control of the strong seed-specific promoter of the gene for the α'-subunit of β-conglycinin (17). Soybean embryos transformed with expression constructs for either CoFADX-1 or CoFADX-2 were found to accumulate several fatty acids that were not detected in untransformed embryos (Fig. 4). The methyl ester of the most abundant of these fatty acids displayed a gas chromatographic retention time identical to that of the calendic acid methyl ester in extracts from *C. officinalis* seeds (Fig. 4). In addition, the mass spectrum of this fatty acid methyl ester was identical to that of methyl calendic acid and was characterized by an abundant molecular ion at m/z 292 (data not shown). To further characterize the identity of this novel fatty acid in soybean embryos transformed with CoFADX-1 or CoFADX-2, fatty acid methyl esters from the transgenic embryos were reacted with MTAD and then analyzed by GC-MS. This reagent readily forms Diels-Alder adducts with conjugated trans,trans-double bonds (21). The product formed from fatty acid methyl esters of the transgenic soybean embryos displayed a mass spectral fragmentation pattern identical to that of the Diels-Alder adduct of calendic acid methyl ester prepared from *C. officinalis* seeds (Fig. 5). As shown by the mass spectra in Fig. 5, the primary adduct detected resulted from derivatization of the trans-Δ⁸- and trans-Δ¹⁰-double bonds of the calendic acid methyl ester, which is consistent with the properties of Diels-Alder reactions (21). These data from transgenic soybean embryos thus demonstrate that CoFADX-1 and CoFADX-2 are associated with the formation of the conjugated trans-Δ⁸- and trans-Δ¹⁰-double bonds of calendic acid.

Two additional fatty acids (corresponding to peaks a and b in Fig. 4B) were detected in low amounts in the transgenic soybean embryos. The methyl ester of peak a in Fig. 4B displayed a mass spectrum identical to that of the methyl ester of calendic acid (data not shown). However, its gas chromatographic retention time on polar phases was slightly longer than that of methyl calendic acid. Based on these properties, this fatty acid was tentatively identified as the trans-Δ⁸,trans-Δ¹⁰,trans-Δ¹²-isomer of calendic acid. The mass spectrum of the fatty acid methyl ester corresponding to peak b in Fig. 4B was characterized by a prominent molecular ion at m/z = 290, which is consistent with that of a methyl 18:4 isomer. Based on substrate feeding studies with *S. cerevisiae* cells expressing CoFADX-1 or CoFADX-2 (described below), this fatty acid was tentatively identified as 18:4trans,10trans,12cis,15cis*, resulting from the activity of these enzymes with α-linolenic acid. These tentatively identified 18:4trans,10trans,12cis,15cis* and 18:4trans,10trans,12cis,15cis* isomers accounted for <0.5% (w/w) and <0.9% (w/w), respectively, of the total fatty acids of the transgenic somatic soybean embryos. The small amounts of these fatty acids in the transgenic plant tissues limited more detailed characterization of their structures.

In somatic soybean embryos expressing CoFADX-2, calendic acid accumulated to as high as 15–22% (w/w) of the total fatty acids (Table I). Surprisingly, this level of calendic acid accumulation had little effect, if any, on the oleic acid (18:1trans,11cis*) content of the transgenic embryos relative to untransformed controls (Fig. 4 and Table I). This result is in marked contrast to previous reports in which the production of unusual fatty acids from FAD2-like enzymes in transgenic seeds was accompanied by large increases in the relative amounts of oleic acid (4, 13, 14). In addition to calendic acid production, the most notable effect on fatty acid composition of soybean embryos expressing CoFADX-2 was a decrease in linoleic acid content compared with untransformed embryos (Table I). This alteration is consistent with linoleic acid serving as the precursor of calendic acid as described below. Small decreases in palmitic acid content were also observed in transgenic embryos expressing either CoFADX-1 or CoFADX-2 (Table I).

**Substrate Specificities of CoFADX-1 and CoFADX-2 in *S. cerevisiae***—CoFADX-1 and CoFADX-2 were expressed in *S. cerevisiae* to examine the substrate specificities of these enzymes. For these experiments, cDNAs encoding CoFADX-1 and CoFADX-2 were introduced behind the GAL1 promoter in the expression vector pESC-URA. In galactose-induced cells transformed with cDNAs for either polypeptide, calendic acid accumulation was observed only when exogenous linoleic acid was included in the growth medium (Fig. 6A). These results thus confirm that linoleic acid is the precursor of calendic acid via the Δ⁸-double bond-modifying activity of CoFADX-1 and
Esters with MTAD, which preferentially reacts with the conjugated 18:4 isomer that contains conjugated double bonds. This fatty acid accounted for \( -1.6\% \) (w/w) of the total fatty acids of yeast cells expressing CoFADX-1 in the presence of exogenous \( a\)-linolenic acid (Table II). The 18:4 isomer was also detected under similar growth conditions in \( S.\) cerevisiae cells expressing CoFADX-2 (data not shown).

To examine the relative activity of CoFADX-1 for linoleic and \( \alpha\)-linolenic acids, cells expressing this enzyme were grown with both fatty acids included in the medium. Although the fatty acids were provided in equal concentrations, \( \alpha\)-linolenic acid was incorporated by cells to amounts nearly twice that of linoleic acid (46\% versus 28\% of the total fatty acids) (Table II). Despite this difference, the accumulation of calendic acid (1.8\% of the total fatty acids), via activity of CoFADX-1 with linoleic acid, was 2-fold greater than the accumulation of 18:4 (0.9\% of the total fatty acids), via activity of CoFADX-1 with \( \alpha\)-linolenic acid (Table II). Similar results were obtained when this experiment was repeated with cells expressing CoFADX-2 (data not shown). These results thus suggest that CoFADX-1 and CoFADX-2 are more active with linoleic acid than with \( \alpha\)-linolenic acid. This observation is consistent with the higher amounts of calendic acid versus 18:4 that accumulated in the transgenic soybean embryos (Fig. 4B) and in yeast cells expressing CoFADX-1 in the presence of either linoleic acid or \( \alpha\)-linolenic acid (Table II). Given the recalcitrant nature of FAD2-type enzymes during purification and in vitro assay (6), determination of more detailed kinetic parameters such as \( K_m\) and \( V_{\text{max}}\) for CoFADX-1 and CoFADX-2 with linoleic and \( \alpha\)-linolenic acids was not attempted.

**DISCUSSION**

We have identified cDNAs for two highly expressed FAD2-related polypeptides (CoFADX-1 and CoFADX-2) from \( C.\) officinalis seed, a tissue that is enriched in calendic acid (18:4, \( \Delta^9\)) and in calendic acid accumulation is similar to what has been previously observed with linoleic acid production in \( S.\) cerevisiae expressing Arabidopsis FAD2 (26). Calendic acid was accounted for as much as 4.5\% (w/w) of the total fatty acids of \( S.\) cerevisiae cells maintained at 16\°C (Table II). Of note, the amount of calendic acid detected in cells expressing CoFADX-1 was at least comparable to that found in cells expressing CoFADX-2 (data not shown).

More detailed characterization of substrate specificity was conducted using \( S.\) cerevisiae cells expressing CoFADX-1. No conjugated dienoic fatty acids were detected in cells grown without exogenous fatty acid or with added oleic acid (data not shown). These results suggest that CoFADX-1 has no or relatively low activity with oleic acid or with the palmitoleic acid (16:1\( \Delta^9\)) found in high levels in cells not provided with exogenous fatty acids.

The inclusion of \( \alpha\)-linolenic acid in the medium resulted in the production of a novel fatty acid by cells expressing CoFADX-1 (Fig. 6D). The methyl ester of this fatty acid displayed a retention time identical to that of peak b in Fig. 4B in gas chromatograms of somatic soybean embryos expressing the divergent \( C.\) officinalis FAD2 enzymes. In addition, the mass spectrum of this fatty acid methyl esters contained an abundant molecular ion at \( m/z = 290\) (data not shown), which is indicative of an 18:4 isomer. Furthermore, reaction of this novel fatty acid methyl ester with MTAD resulted in the formation of a Diels-Alder adduct (as determined by GC-MS) with a molecular ion at \( m/z = 403\) (data not shown), which is consistent with an 18:4 isomer that contains conjugated double bonds.

Given these results and our demonstration that CoFADX-1 and CoFADX-2 convert the \( \text{cis-}\Delta^9\)-double bond of linoleic acid into \( \text{trans-}\Delta^8\) and \( \text{trans-}\Delta^{10}\)-double bonds, the 18:4 isomer formed from \( \alpha\)-linolenic acid (18:3\( \Delta^9,\Delta^{12},\Delta^{15}\)) is probably 18:4\( \Delta^{12}\), \( \text{trans-}\Delta^{10}\), \( \text{trans-}\Delta^8\), and \( \text{cis-}\Delta^{15}\). This fatty acid accounted for \( -1.6\%\) (w/w) of the total fatty acids of yeast cells expressing CoFADX-1 in the presence of exogenous \( a\)-linolenic acid (Table II). The 18:4 isomer was also detected under similar growth conditions in \( S.\) cerevisiae cells expressing CoFADX-2 (data not shown).
Tentatively identified as the methyl ester of 18:4 Δ9,12

C. officinalis with linoleic or α-linolenic acid. The gas chromatograms shown in A and C were derived from S. cerevisiae cells containing only the expression vector pESC-URA and grown in medium containing linoleic or α-linolenic acid, respectively. The gas chromatograms shown in B and D were derived from cells expressing the CoFADX-1 cDNA in vector pESC-URA and grown in medium containing linoleic or α-linolenic acid, respectively. Shown in E are fatty acid methyl esters prepared from C. officinalis seeds. The peak labeled 18:4 in D was tentatively identified as the methyl ester of 18:4 Δ9,12,15,18ω6c. Peaks labeled with numbers represent methyl esters of the following fatty acids: peak 1, palmitic acid (16:0); peak 2, palmitoleic acid (16:1 Δ9ω6c); peak 3, stearic acid (18:0); peak 4, oleic acid (18:1 Δ9ω6c); peak 5, linoleic acid (18:2 Δ9,12ω6c); and peak 6, α-linolenic acid (18:3 Δ9,12,15ω6c).

FADX-1 and CoFADX-2 may provide useful comparative information for understanding the structural basis of regiospecificity in FAD2-type enzymes. In this regard, the amino acid sequences of these enzymes contain several insertions and deletions relative to other FAD2-type enzymes (Fig. 1). It is interesting to speculate that these structural features may be associated with the variant regiospecificities of CoFADX-1 and CoFADX-2.

It should be noted that a cDNA for a FAD2-related enzyme (CoDES) from C. officinalis was recently identified by Fritsche et al. (28) and was reported to be a calendic acid-producing desaturase. Note that CoDES is identified as CoFad2 (76% identity) (24). In addition, we were unable to detect any copies of cDNAs for CoDES in the —3000 random cDNAs that were sequenced from S. officinalis seeds. In contrast, cDNAs for CoFADX-1 and CoFADX-2 accounted for —23% of the C. officinalis seed ESTs. Of note, we have identified expressed genes encoding FAD2-related polypeptides that share 80–90% amino acid sequence identity with CoDES in a variety of other Asteraceae species, including those that do not accumulate calendic fatty acids in their seed oils. Thus, it was unlikely that CoDES encodes a seed fatty acid conjugase.

Among the unexpected results from the transgenic production of calendic acid in somatic soybean embryos was the lack of an accompanying high oleic acid phenotype. This finding is in contrast to that previously observed with unusual fatty acid synthesis resulting from the transgenic expression of divergent
FAD2 enzymes, including the castor and Lesquerella hydroxylases and the Momordica Δ12-conjugase (4, 13, 14). Intransgenic seeds and somatic soybean embryos that express these enzymes, the accumulation of unusual fatty acids is typically accompanied by 2–4-fold increases in the relative content of oleic acid (4, 13, 14). In contrast to calandric acid, unusual fatty acids such as ricinoleic acid (12-OH:18:1Δ9cis) and α-eleostearic acid (18:3Δ9cis,11trans,13trans) result from chemical modifications of the Δ12-position of the C18 fatty acid chain. It is thus possible that unusual fatty acids with modifications of the Δ12-position directly or indirectly inhibit oleic acid desaturation on phosphatidylcholine in transgenic seeds or embryos. This inhibition apparently does not occur or is more limited with the transgenic production of fatty acids that have similar modifications of the Δ9-position.

Another unexpected result from the transgenic somatic soybean embryos was the accumulation of small amounts of an 18:4 isomer that we tentatively identified as 18:4Δtrans,10trans,12cis,15cis. Based on expression studies with S. cerevisiae, this fatty acid results from the modification of the Δ9-double bond of α-linolenic acid by CoFADX-1 or CoFADX-2 activity. To our knowledge, the occurrence of 18:4 in seeds has not been previously reported. The lack of detectable amounts of calendic acid resulting from the expression of CoFADX-1 and CoFADX-2 were as high as 4% (w/w) regardless of tissue. Differences in levels of calendic acid accumulation in these lines may be due to factors associated with the transgenic tissues. Differences in levels of calendic acid accumulation in these lines may be due to factors associated with the transgenic tissues.

Amounts of calandric acid in somatic soybean embryos expressing CoFADX-1 and CoFADX-2 were as high as 4% (w/w) and 22% (w/w), respectively, of the total fatty acids of these tissues. Differences in levels of calandric acid accumulation in these lines may be due to factors associated with the transgenic expression of CoFADX-1 and CoFADX-2 or to a lower specific activity of CoFADX-1 in the transgenic soybean embryos. Regardless, amounts of calandric acid resulting from the expression of either CoFADX-1 or CoFADX-2 are likely sufficient to increase the oxidation rate of triacylglycerols in the transgenic soybean embryos. In this regard, seed oils such as tung oil that are enriched in polyunsaturated fatty acids with conjugated double bonds are used as drying agents in coating materials (e.g., paints, varnishes, and inks) because of their high rates of oxidation (2, 3). Therefore, the transgenic expression of CoFADX-1 or CoFADX-2 may ultimately be useful for the production of improved drying oils in existing oilseed crops such as soybean.

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