Dimorphotheca is a genus of plants that produces unusual fatty acids, which are found in plant fatty acids, including a C-9 hydroyx group, Δ^{10}\Delta^{12}-conjugated double bonds, and Δ^{9}\Delta^{12} unsaturation. These fatty acids are not typically found in plant fatty acids, including a C-9 hydroyx group, Δ^{10}\Delta^{12}-conjugated double bonds, and Δ^{9}\Delta^{12} unsaturation. 

Expressed sequence tag analysis was conducted to determine the biosynthetic origin of dimorphothecic acid. cDNAs for two divergent forms of Δ^{12}\oleic acid desaturase, designated DsFAD2-1 and DsFAD2-2, were identified among expressed sequence tags generated from developing Dimorphotheca sinuata seeds. Expression of DsFAD2-1 in Saccharomyces cerevisiae and soybean embryos, DsFAD2-2 converted 18:2Δ^{cis,12} into dimorphothecic acid. When co-expressed with DsFAD2-1 in soybean embryos or yeast, DsFAD2-2 converted 18:2Δ^{cis,12} into dimorphothecic acid. When co-expressed with DsFAD2-1 in soybean embryos or yeast, DsFAD2-2 converted 18:2Δ^{cis,12} into dimorphothecic acid. When co-expressed with DsFAD2-1 in soybean embryos or yeast, DsFAD2-2 converted 18:2Δ^{cis,12} into dimorphothecic acid.
Expression of DsFAD2-1 in Saccharomyces cerevisiae—For expression in somatic embryos of soybean (Glycine max (L.) Merril cv. Jack), the cDNAs for DsFAD2-1 and DsFAD2-2 were linked to promoter for the α-subunit of β-conglycinin in the previously described vector pKS17 (14). This soybean expression vector is identical to pKS67 except that it lacks a hygromycin resistance marker for selection of transgenic events. Gene fusions of the DsFAD2-1 or DsFAD2-2 cDNAs with the β-conglycinin promoter and phaseolin 3’ non-translated region in vector pKS67 were introduced into soybean somatic embryos by using particle bombardment (15). Experiments were also conducted in which the DsFAD2-2 cDNA in pKS67 was co-transformed with the DsFAD2-1 cDNA in pKS17. In these experiments, the DsFAD2-2-containing plasmids were co-transformed at a molar ratio of 10:1 (DsFAD2-1:DsFAD2-2) of the two expression constructs. A similar co-transformation methodology has been reported previously (14). Transgenic embryos were selected by hygromycin resistance conferred by the marker gene for hygromycin phosphotransferase in pKS67. Hygromycin-resistant embryos were propagated to maturity, and expression of the DsFAD2-1 and DsFAD2-2 transgenes was confirmed by using previously described protocols (6, 9).

Fatty Acid Analysis of S. cerevisiae and Soybean Somatic Embryos—Fatty acid methyl esters were prepared from S. cerevisiae cultures by direct transesterification of cell pellets in sodium methoxide/methanol (6). The fatty acid methyl esters were then analyzed by gas chromatography (GC) using an Agilent 6890 chromatograph fitted with a DB-23 column (30-m × 0.25-mm inner diameter, 0.25-μm film; Agilent). The oven temperature was programmed from 185 °C (2-min hold) to 225 °C at 5 °C/min, and eluted fatty acid methyl esters were detected by flame ionization. The retention time of the 18:2 methyl ester produced by DsFAD2-1-expressing cells was compared with that of a standard mixture of cis-trans isomers of 18:2Δ9,12 methyl ester (Sigma). In addition, structural analysis of fatty acid methyl esters from yeast extracts was conducted by use of GC-mass spectrometry (MS) as described below for the analysis of soybean fatty acid methyl esters.

The double bond positions of 18:2 and 18:3 isomers produced in S. cerevisiae were determined by GC-MS following derivatization of fatty acids to diethylamide derivatives. Free fatty acids were initially prepared by saponification of cell pellets from S. cerevisiae cultures (grown as described above) in 1 ml of 0.6 N potassium hydroxide in methanol. Following a 1-h incubation at 70 °C, free fatty acids were extracted by partitioning the reaction with the addition of 0.9 ml of 1 N hydrochloric acid. A full-length fatty acid fraction was then subjected to the GC-MS analysis following derivatization with N-methyl-N-(trimethylsilyl)trifluoroacetamide. The derivatized fatty acid methyl esters were analyzed by GC-MS using an HP6890 interfaced with a HP5973 (Agilent) mass selective detector. Sample components were resolved with a DB-23 capillary column and the oven temperature was programmed from 255 °C (2-min hold) to 235 °C at 5 °C/min.

Fatty acid methyl esters were prepared from soybean somatic embryos by transesterification in 1% (v/v) sodium methoxide in methanol as described previously (6, 9). The recovered fatty acid methyl esters...
were analyzed by GC with an Omegawax 320 column (30-m \times 0.32-mm inner diameter; Supelco). The oven temperature was programmed from 185 °C (4-min hold) to 215 °C at a rate of 5 °C/min and then to 240 °C at 20 °C/min (1-min hold). Fatty acid methyl esters were also analyzed by GC-MS using the instrument described above fitted with an HP-INNO-Wax column. The oven temperature was programmed from 180 °C (3.5-min hold) to 215 °C at a rate of 2 °C/min (2-min hold) and then to 230 °C at 10 °C/min. For analyses of yeast cells and soybean embryos transformed with DsFAD2-2, the recovered fatty acid methyl esters were dried under nitrogen and reacted with 50 µl of the silylating reagent bis(tri-methylsilyl)trifluoroacetamide:trimethylchlorosilane (99:1, v/v) (Supelco) to convert the hydroxy group of dimorphecolic acid to a trimethylsilyl (TMS) ether derivative for GC and GC-MS analyses (11). Samples were incubated at 70 °C for 30 min. The samples were then dried under nitrogen and resuspended in heptane for GC and GC-MS analyses as described above.

Of note, our identification of cis-trans orientations of Δ₁₂ double bonds of 16:2 and 18:2 methyl esters (as described under “Results”) was consistent with the known chromatographic properties of the different GC columns used in the analyses of S. cerevisiae and soybean extracts. For example, the trans isomer of a given fatty acid methyl ester elutes prior to the cis isomer on the 50% cyanopropyl, methylpolysiloxane phase of a DB-23 column (17) as was observed in the analysis of S. cerevisiae extracts (see Fig. 2). Conversely a cis isomer elutes before the corresponding trans isomer on the polyethylene glycol phase of OmegaWax 320 and HP-INNO-Wax columns (17) as was observed in the analysis of soybean extracts (see Fig. 3).

RESULTS

Identification of Two Divergent FAD2 Polypeptides in D. sinuata Seeds—The enzymes associated with the synthesis of dimorphecolic acid have not been previously identified, and little direct evidence has been presented for its biosynthetic pathway. To provide clues for the biosynthetic origin of dimorphic acid, an EST analysis of developing D. sinuata seeds was conducted. From the sequences of 2,669 randomly selected cDNAs, 12 ESTs for FAD2-like polypeptides were identified. This was of particular interest because divergent members of the FAD2 family catalyze the synthesis of a number of unusual fatty acids, including those that, like dimorphic acid, contain hydroxy groups and conjugated double bonds (18). The 12 D. sinuata ESTs included five ESTs that appeared to encode a functionally divergent form of FAD2, designated DsFAD2-1, and three ESTs that appeared to encode a second divergent form of FAD2, designated DsFAD2-2. The remaining four ESTs appeared to encode typical cis-Δ₁₂-oleic acid desaturases. The putative identification of D. sinuata FAD2 ESTs as functionally “typical” or “divergent” was based on properties of their deduced amino acid sequences. The primary structures of FAD2s contain three His-rich domains or “boxes” that are believed to coordinate active site diiron atoms (18, 19). The consensus sequence of the first His box is HECGGH. In all members of the FAD2 family that function as cis-Δ₁₂-oleic acid desaturases, this box is preceded by Ala. However, all known enzymes of this family that contain Gly in this position catalyze alternative reactions such as fatty acid hydroxylation and epoxygenation (20–22). The amino acid sequences of both DsFAD2-1 and DsFAD2-2 contain a Gly residue preceding the first His box. Based on this, we hypothesized that DsFAD2-1 and DsFAD2-2 do not function as typical cis-Δ₁₂-oleic acid desaturases. The other four D. sinuata ESTs identified as cis-Δ₁₂-oleic acid desaturases had Ala preceding the first His box and other sequence elements consistent with the cis-Δ₁₂-oleic acid desaturase functional class of FAD2 enzymes (23).

Interestingly the amino acid sequences of DsFAD2-1 and DsFAD2-2 share only 48% identity. DsFAD2-1 is most closely related to a cis-Δ₁₂-oleic acid desaturase from Helianthus annuus and shares ~60–75% amino acid sequence identity with all known cis-Δ₁₂-oleic acid desaturases. This polypeptide also shares 63–67% identity with FAD2-type fatty acid hydroxylases and Δ₁₂-fatty acid conjugases from Aeluropus lortorii (23) and Punica granatum (24) but shares ≤55% identity with FAD2 epoxygenases, acetylenases, and Δ₁₂-fatty acid conjugases. In addition, the amino acid sequence of DsFAD2-1 does not display a distinct phylogenetic relationship with those of any specific FAD2 functional class (Fig. 1). As a result, it was not possible to predict the enzymatic function of DsFAD2-1 from its primary structure alone. DsFAD2-2, by contrast, shares ≤55% identity with all members of the FAD2 family except the Δ₁₂-fatty acid conjugases from Calendula officinalis (6, 7). DsFAD2-2 and the C. officinalis Δ₁₂-fatty acid conjugases share ~75% amino acid sequence identity, and these polypeptides display a close phylogenetic relationship (Fig. 1). The
ester from DsFAD2-1-expressing cells instead displayed a retention time identical to that of methyl 18:2Δ9cis,12trans in a standard mixture of methyl 18:2 isomers (Fig. 2D). Based on these chromatographic and mass spectral data, it was concluded that DsFAD2-1 functions as trans-Δ12 desaturase, and when expressed in S. cerevisiae, this enzyme is able to convert oleic acid (18:1Δ9cis) into 18:2Δ9cis,12trans. It is presumed that the 16:2 formed by DsFAD2-1 is the Δ9cis,12trans isomer that arises from the trans-Δ12 desaturation of palmitoleic acid (16:1Δ9cis). Although Δ9cis,12trans isomers were the predominant form of 16:2 and 18:2 in cells expressing DsFAD2-1 (Table I), Δ9cis,12trans isomers of these fatty acids were detectable at amounts of <0.2% of the total fatty acids of yeast extracts. This observation suggests that DsFAD2-1 may have a very limited ability to also catalyze cis-Δ12 desaturation.

Consistent with the results obtained from yeast, expression of DsFAD2-1 in soybean somatic embryos yielded a novel 18:2 isomer (Fig. 3B). The methyl ester of this isomer displayed a GC retention time that was identical to that of a methyl 18:2 isomer from D. sinuata seed extracts. This isomer has previously been identified as the 18:2Δ9cis,12trans (4) (Fig. 3C). The 18:2Δ9cis,12trans isomer accounted for 15% of the fatty acids of embryos transformed with the DsFAD2-1 cDNA (Table I). In addition to the production of 18:2Δ9cis,12trans, the expression of DsFAD2-1 resulted in a reduction in the content of the 18:2Δ9cis,12cis isomer relative to the nontransformed embryos (Table II). This was likely due to competition of DsFAD2-1 with the native cis-Δ12-oleic acid desaturase of soybean embryos for the available pool of oleic acid, the substrate for both enzymes. Overall the results from yeast and soybean expression indicate that DsFAD2-1 functions primarily as a trans-Δ12-oleic acid desaturase for the conversion of 18:1Δ9cis into 18:2Δ9cis,12trans.

Functional Characterization of DsFAD2-2—The function of DsFAD2-2 was initially characterized by expression in soybean somatic embryos. Fatty acid methyl esters prepared from the soybean embryos were reacted with a silylating reagent to facilitate the GC analysis of any hydroxy fatty acids, such as dimorphecolic acid, that might be formed by DsFAD2-2 activity. This reagent converts hydroxyl residues into TMS-ether derivatives. The mass spectrum of the TMS derivative of dimorphecolic acid methyl ester has an abundant 225 m/z ion. This diagnostic ion results from fragmentation at the C-8 and C-9 atoms (Fig. 4A). Using GC-MS, the 225 m/z ion can be extracted from total ion chromatograms of soybean embryo fatty acid methyl esters to provide a very sensitive means of detecting dimorphecolic acid production. Through the use of this method, two fatty acid methyl esters with mass spectra identical to that of the TMS derivative of methyl dimorphoco-
late were detected in extracts from soybean embryos expressing DsFAD2-2 (Fig. 5A). Neither fatty acid methyl ester was detected in extracts from non-transformed soybean embryos. The less abundant of the two peaks from DsFAD2-2-expressing embryos had a retention time identical to that of the TMS derivative of methyl dimorphecolate from D. sinuata seeds (Fig. 5A and C). The major peak had the same retention time as the TMS derivative of the cis-Δ12 isomer of methyl dimorphecolate (9-OH-18:2Δcis,12Δcis). This fatty acid has been previously identified as a very minor component of the seed oil of D. sinuata (27). Although these isomers of dimorphecolic acid were detectable in soybean embryo extracts, the cis-Δ12 isomer comprised <0.1% of the total fatty acids, and dimorphecolic acid (i.e., the trans-Δ12 isomer) comprised <0.05% of the total fatty acids. These results indicated that DsFAD2-2 catalyzes the formation of the C-9 hydroxyl group and the trans-Δ10 double bond of dimorphecolic acid. These structural features likely resulted from modification of the Δ9 double bond of linoleic acid isomers based on the sequence homology of DsFAD2-2 with Δ9-fatty acid conjugases from C. officinalis.

We hypothesized that the cis-Δ12 isomer of dimorphecolic acid is formed by DsFAD2-2 activity with cis-Δ12-linoleic acid, the major fatty acid of somatic soybean embryos. Conversely, the trans-Δ12 double bond of dimorphecolic acid in D. sinuata seeds arises from DsFAD2-2 activity with trans-Δ12-linoleic acid, the product of DsFAD2-1. To test this hypothesis, DsFAD2-1 and DsFAD2-2 were co-expressed in soybean somatic embryos. In embryos that produced trans-Δ12-linoleic acid via DsFAD2-1 activity, dimorphecolic acid, rather than its cis-Δ12 isomer, accounted for ~90% of the total dimorphecolic acid content (Figs. 4B and 5B). In addition, dimorphecolic acid isoforms accounted for ~0.5–1.0% of the total fatty acids of the somatic embryos, which was a 5–10-fold increase relative to embryos that expressed DsFAD2-2 alone. These results were consistent with the biosynthesis of dimorphecolic acid by activity of DsFAD2-2 with trans-Δ12-linoleic acid produced by DsFAD2-1. Of note, the detection of dimorphecolic acid at amounts of <0.05% of the fatty acids in embryos that express only DsFAD2-2 (see Fig. 5B) suggests that trace amounts of trans-Δ12-linoleic acid naturally occur in soybean somatic embryos.

The involvement of DsFAD2-1 and DsFAD2-2 in dimorphecolic acid synthesis was examined further by co-expression of these enzymes in S. cerevisiae. This system is particularly advantageous for examining the coordinate activities of DsFAD2-1 and DsFAD2-2 because it does not normally produce linoleic acid. Consistent with the results obtained from soybean somatic embryos, co-expression of DsFAD2-1 and DsFAD2-2 in S. cerevisiae was accompanied by the production of dimorphecolic acid (Fig. 6A). This fatty acid was not detected in cells harboring the empty expression vector or in cells transformed with only the DsFAD2-2 cDNA (Fig. 6C). Dimorphecolic acid accounted for ~0.5% of the fatty acids of the yeast cells expressing DsFAD2-1 and DsFAD2-2. In addition, a fatty acid with a

| Fatty acid | Non-transformed (n = 3) | +DsFAD2-1 (n = 5) |
|------------|-------------------------|------------------|
| 16:0       | 13.6 ± 1.2              | 13.3 ± 0.4       |
| 18:0       | 2.6 ± 0.3               | 2.6 ± 0.4        |
| 18:1Δcis   | 7.5 ± 0.4               | 8.7 ± 2.6        |
| 18:1Δitra  | 1.7 ± 0.2               | 3.5 ± 0.1        |
| 18:2Δcis,12Δcis | 55.6 ± 1.2       | 38.8 ± 1.7       |
| 18:2Δitra,12Δitra | ND*               | 15.0 ± 4.1       |
| 18:3Δitra,12Δitra,15Δitra | 18.1 ± 1.0  | 17.4 ± 3.0       |
| Other†     | ≤1.0                    | ≤1.2             |

* ND, not detected.
† Includes 20:0, 20:1, 22:0, and 22:1.
mass spectrum consistent with that of 9-OH-16:2\(\Delta_{12}^{cis,12trans}\) was detected in these cells, presumably from the activity of DsFAD2-2 with the 16:2\(\Delta_{12}^{cis,12trans}\) product of DsFAD2-1 (results not shown). The mass spectrum of the TMS-derivatized methyl ester of this fatty acid contained a molecular ion of 354 \(m/z\) and an abundant 197 \(m/z\) ion from fragmentation between the C-8 and C-9 atoms. It is notable that no 9-OH-18:2\(\Delta_{12}^{cis,12trans}\) was detected in cells that expressed only DsFAD2-2. The lack of this product indicates that DsFAD2-2 does not function on oleic acid and that DsFAD2-2 activity occurs after the production of trans-\(\Delta_{12}^{cis}\)-linoleic acid by DsFAD2-1.

DsFAD2-2 was also co-expressed in S. cerevisiae with a typical cis-\(\Delta_{12}^{cis}\)-oleic acid desaturase from E. lagascae to assess the

**Fig. 5.** Selected ion chromatograms from GC-MS analyses of TMS-derivatized fatty acid methyl esters from S. cerevisiae co-expressing DsFAD2-2 and DsFAD2-1 (A), co-expressing DsFAD2-2 and a cis-\(\Delta_{12}^{cis}\)-oleic acid desaturase (B), or expressing DsFAD2-2 alone (C). Chromatograms were obtained by extracting the 225 \(m/z\) ion (diagnostic for the TMS derivative of methyl dimorphecolate) from total ion chromatograms of TMS-derivatized fatty acid methyl esters. The cis-\(\Delta_{12}^{cis}\) isomer of dimorphecolic acid (9-OH-18:2\(\Delta_{12}^{cis,12trans}\)) and dimorphecolic acid (9-OH-18:2\(\Delta_{12}^{cis,12trans}\)) accounted for  0.1% and 0.05%, respectively, of the total fatty acids of soybean embryos expressing DsFAD2-2 alone (A). Dimorphecolic acid comprised 1% of the total fatty acids from soybean embryos that co-expressed DsFAD2-1 and DsFAD2-2 (B). The trans-\(\Delta_{12}^{cis}\) isomer of linoleic acid, formed by DsFAD2-1 activity, also comprised 10% of the total fatty acids of these embryos.

**Fig. 6.** Selected ion chromatograms from the GC-MS analyses of TMS-derivatized fatty acid methyl esters from S. cerevisiae co-expressing DsFAD2-2 and DsFAD2-1 (A), co-expressing DsFAD2-2 and a cis-\(\Delta_{12}^{cis}\)-oleic acid desaturase (B), or expressing DsFAD2-2 alone (C). Chromatograms were obtained by extracting the 225 \(m/z\) ion (diagnostic for the TMS derivative of methyl dimorphecolate) from total ion chromatograms of TMS-derivatized fatty acid methyl esters. The cis-\(\Delta_{12}^{cis}\) isomer of dimorphecolic acid (9-OH-18:2\(\Delta_{12}^{cis,12trans}\)) and dimorphecolic acid (9-OH-18:2\(\Delta_{12}^{cis,12trans}\)) accounted for  0.1% and 0.05%, respectively, of the total fatty acids of soybean embryos expressing DsFAD2-2 alone (A). Dimorphecolic acid comprised 1% of the total fatty acids from soybean embryos that co-expressed DsFAD2-1 and DsFAD2-2 (B). The trans-\(\Delta_{12}^{cis}\) isomer of linoleic acid, formed by DsFAD2-1 activity, also comprised 10% of the total fatty acids of these embryos.
Dimorpheolic Acid Biosynthesis

![Diagram of dimorpheolic acid biosynthesis]

**Fig. 7. Proposed biosynthetic pathway of dimorpheolic acid in *D. sinuata* seeds.** The expression studies outlined are consistent with a biosynthetic pathway of dimorpheolic acid involving the **trans**-\(\Delta^{12}\) desaturation of oleic acid by the activity of DsFAD2-1 to form **trans**-\(\Delta^{12}\)-linoleic acid. The \(\Delta^{10}\) double bond of **trans**-\(\Delta^{12}\)-linoleic acid is then converted into a 9-OH group and **trans**-\(\Delta^{10}\) double bond by the activity of DsFAD2-2 to form dimorpheolic acid. The fatty acid substrates for each reaction are likely bound to a phospholipid (as indicated by **R**) as has been shown for other FAD2-catalyzed biosynthetic pathways (19).

**DISCUSSION**

In this study, two divergent forms of FAD2, designated DsFAD2-1 and DsFAD2-2, were identified among ESTs from *D. sinuata* seeds, which accumulate high levels of the unusual fatty acid dimorpheolic acid. DsFAD2-1 was shown to be a **trans**-\(\Delta^{12}\)-oleic acid desaturase by expression in yeast and soybean somatic embryos. Using a similar approach, DsFAD2-2 was demonstrated to catalyze the conversion of the **trans**-\(\Delta^{10}\) double bond of linoleic acid isomers into a C-9 hydroxyl group and **trans**-\(\Delta^{10}\) double bond. In addition, dimorpheolic acid biosynthesis was conferred to yeast and soybean somatic embryos when DsFAD2-1 and DsFAD2-2 were co-expressed. These results thus provide conclusive evidence that DsFAD2-1 and DsFAD2-2 function in a coordinate manner to produce dimorpheolic acid in *D. sinuata* seeds. This, to our knowledge, is the first report of the evolution of two divergent FAD2s for the production of an unusual fatty acid in seeds of a single species.

The function of DsFAD2-1 as primarily a **trans**-\(\Delta^{12}\)-oleic acid desaturase appears to be novel relative to that of previously characterized members of the FAD2 family. Expression of DsFAD2-1 in yeast resulted in the production of **trans**-\(\Delta^{12}\)-linoleic acid and only traces amounts of **cis**-\(\Delta^{12}\)-linoleic acid. Similarly expression of this enzyme in soybean somatic embryos was accompanied by the accumulation of **trans**-\(\Delta^{12}\)-linoleic acid to \(-15\%\) of the total fatty acids. No other unusual fatty acid products were detected in the transgenic embryos. The **Delta**-12 fatty acid conjugase from *A. fordii* seeds is the only other member of the FAD2 family that has been reported to display **trans**-\(\Delta^{12}\)-oleic acid desaturase activity. Dyer et al. (23) have recently shown that small amounts of **trans**-\(\Delta^{12}\)-linoleic acid accumulate when this enzyme is expressed in yeast. In contrast to DsFAD2-1, however, **trans**-\(\Delta^{12}\)-oleic acid desaturation is a minor activity of the *A. fordii* **Delta**-12 fatty acid conjugase in planta. The seed oil of *A. fordii* contains 80% eleostearic acid from activity of the **Delta**-12-fatty acid conjugase but \(<1\% \text{ **trans**-\(\Delta^{12}\)linoleic acid from the alternative activity of this enzyme (23).**

DsFAD2-1 and **cis**-\(\Delta^{12}\)-oleic acid desaturases likely have very similar catalytic mechanisms but differ in their substrate binding properties. In this regard, the sphingolipid **Delta**-desaturase of plants has been shown to catalyze both **cis** and **trans** desaturation of sphingolipid long chain bases (28). It was proposed that the different double bond orientations arise from the stereochemical conformation in which the acyl substrate is presented to the diiron atoms in the active site (18). Based on this proposal, **trans**-\(\Delta^{12}\) desaturation of oleic acid occurs when the C-12 and C-13 atoms are presented to the diiron center in a **trans** conformation, and **cis**-\(\Delta^{12}\) occurs when these atoms are presented in the **cis** conformation. It is therefore likely that DsFAD2-1 has evolved amino acid substitutions that alter the conformation of the fatty acid substrate in the active site relative to **cis**-\(\Delta^{12}\)-oleic acid desaturases. Apart from differences in the geometry of substrate binding, removal of hydrogen atoms from acyl chains probably occurs through the same active site chemistry in **cis**- and **trans**-**Delta**-12-oleic acid desaturases.

DsFAD2-2 is most closely related to **Delta**-9 fatty acid conjugases from *C. officinalis*, which catalyze the synthesis of calendic acid (18;**Delta**-9**trans**-10**trans**, 12-cis), DsFAD2-2 shares \(<75\%\) amino acid sequence identity with these enzymes. DsFAD2-2, like the **Delta**-9 fatty acid conjugases, modifies the **Delta**-9 double bond of linoleic acid. In addition, both types of enzymes generate a **trans**-**Delta**-10 double bond. The *C. officinalis* **Delta**-9 fatty acid conjugases have recently been shown to catalyze the removal of hydrogen atoms from the C-8 and C-11 positions that flank the **Delta**-9 double bond of linoleic acid (8). Removal of a hydrogen atom from the C-11 position appears to be the initial step in the catalytic mechanism of the **Delta**-9 fatty acid conjugases (8). It is likely that DsFAD2-2 operates through a mechanism that is similar to that of the **Delta**-9 fatty acid conjugases as well as desaturase-related fatty acid hydroxylases (29, 30). For example, DsFAD2-2-catalyzed removal of a hydrogen atom from the C-11 position may result in an intermediate that contains a radical on the C-9 atom (Fig. 8). This radical may then remove an oxygen atom from the diiron center because of its close proximity to the catalytic core of DsFAD2-2. The end result of this mechanism would be a C-9 hydroxyl group and a **trans**-**Delta**-10 double bond as found in dimorpheolic acid (Fig. 8). Such a variation on the mechanism of the **Delta**-9-fatty acid conjugases would be possible if
there is a slight difference in the positioning of the fatty acid substrate relative to the diiron center in DsFAD2-2. This difference could arise from relatively small alterations in the conformation of the active site of DsFAD2-2 relative to the Δ^3-fatty acid conjugases. It is notable that the Δ^3-fatty acid conjugases and DsFAD2-2 have slightly different substrate specificities. The Δ^3-fatty acid conjugases are most active with cis-Δ^{12}-linoleic acid (7, 8), whereas DsFAD2-2 appears to be most active with trans-Δ^{12}-linoleic acid. For example, soybean somatic embryos that co-express DsFAD2-1 and DsFAD2-2 contain –35% cis-Δ^{12}-linoleic acid and 15% trans-Δ^{12}-linoleic acid. Yet DsFAD2-2 uses trans-Δ^{12}-linoleic acid to the near exclusion of the cis-Δ^{12} isomer for the synthesis of dimorpho- 

colic acid.

The identification of DsFAD2-1 and DsFAD2-2 extends the range of functional outcomes that have been ascribed to the FAD2 family of enzymes. The amino acid sequences of DsFAD2-1 and DsFAD2-2 will likely be useful for structure-function studies of FAD2 and other membrane-associated diiron-oxo enzymes. In addition, the availability of genes for DsFAD2-1 and DsFAD2-2 will facilitate biotechnological studies aimed at producing high levels of dimorphoic acid in microbes and plants for industrial applications. Our demonstration that dimorphoic acid biosynthesis can be conferred to yeast and soybean somatic embryos by co-expression of DsFAD2-1 and DsFAD2-2 lays the groundwork for such studies.

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