Stearoyl-Acyl Carrier Protein and Unusual Acyl-Acyl Carrier Protein Desaturase Activities Are Differentially Influenced by Ferredoxin[1]

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Acyl-acyl carrier protein (ACP) desaturases function to position a single double bond into an acyl-ACP substrate and are best represented by the ubiquitous Δ9 18:0-ACP desaturase. Several variant acyl-ACP desaturases have also been identified from species that produce unusual monoenoic fatty acids. All known acyl-ACP desaturase enzymes use ferredoxin as the electron-donating cofactor, and in almost all previous studies the photosynthetic form of ferredoxin rather than the non-photosynthetic form has been used to assess activity. We have examined the influence of different forms of ferredoxin on acyl-ACP desaturases. Using combinations of in vitro acyl-ACP desaturase assays and [^{14}C]malonyl-coenzyme A labeling studies, we have determined that heterotrophic ferredoxin isoforms support up to 20-fold higher unusual acyl-ACP desaturase activity in coriander (*Coriandrum sativum*), *Thunbergia alata*, and garden geranium (*Pelargonium* × *hortorum*) when compared with photosynthetic ferredoxin isoforms. Heterotrophic ferredoxin also increases activity of the ubiquitous Δ9 18:0-ACP desaturase 1.5- to 3.0-fold in both seed and leaf extracts. These results suggest that ferredoxin isoforms may specifically interact with acyl-ACP desaturases to achieve optimal enzyme activity and that heterotrophic isoforms of ferredoxin may be the in vivo electron donor for this reaction.

Acyl-acyl carrier protein (ACP) desaturases are a class of soluble enzymes that function to add a double bond to an acyl group esterified to ACP. The first acyl-ACP desaturase to be recognized and studied was the stearoyl-ACP desaturase (Δ9 18:0-ACP desaturase) in Euglena (*Euglena gracilis*) (Nagai and Bloch, 1968). Δ9 18:0-ACP desaturase activity was also identified in higher plants (Jacobson et al., 1974), and the first clones were reported for castor (*Ricinus communis*) seed and cucumber by Shanklin and Somerville (1991) and for safflower by Thompson et al. (1991). Several cDNA clones encoding various "unusual acyl-ACP desaturases" have more recently been identified. These enzymes differ from the ubiquitous Δ9 18:0-ACP desaturase in the chain length of the substrate and/or the position of double bond insertion (Shanklin and Cahoon, 1998). To date, unusual acyl-ACP desaturases have been isolated from coriander (*Coriandrum sativum*; Δ4 16:0-ACP desaturase), *Thunbergia alata* (Δ6 16:0-ACP), garden geranium (*Pelargonium* × *hortorum*; Δ9 14:0-ACP), milkweed (Δ9 16:0-ACP), and cat's claw (Δ9 16:0-ACP desaturase) (Cahoon et al., 1992, 1997a, 1998; 1994; Schultz et al., 1996). Aided by the crystal structure of the Δ9 18:0-ACP desaturase from castor seed (Lindqvist et al., 1996) and sequence comparisons of the numerous acyl-ACP desaturases with altered functions, Cahoon et al. (1997b, 1998) have further defined amino acids residues that are involved in substrate specificity and double bond placement.

The discovery of the unusual acyl-ACP desaturases has led to the realization of the potential applications of these genes to modify plant oils for production of industrially useful monoenoic oils. However, in all cases tested thus far, production of expected monoenes resulting from expression of the unusual acyl-ACP desaturases has led to only low levels of the unusual monoenes (Cahoon et al., 1992; D.J. Schultz, M.C. Suh, and J.B. Ohlrogge, unpublished data). One potential limiting factor to unusual monoene production in transgenic plants is the source of reducing electrons supplied by ferredoxin.

Initial biochemical characterization of the Δ9 18:0-ACP desaturase indicated that ferredoxin functions as the electron donor (Nagai and Bloch, 1966, 1967, 1968). Further characterizations demonstrated that ferredoxin-reducing systems, dependent on either NADPH/ferredoxin NADP$^+$ reductase (FNR) or on photoreduction of ferredoxin supplied with chloroplast lamellae, could provide electrons to the desaturase reaction (Jacobson et al., 1974).

Ferredoxin has been most extensively studied in photosynthetic tissue where this protein serves as a major electron carrier from photosystem I to produce NADPH. Early analysis of ferredoxins in plants showed that at least two leaf ferredoxin isoforms exist (Sakihama and Shin, 1987) and recently a more

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complex picture of ferredoxins has emerged. Multiple ferredoxin isoforms are now known to exist in both photosynthetic and non-photosynthetic tissues (Wada et al., 1989; Morigasaki et al., 1990; Green et al., 1991; Hase et al., 1991a, 1991b; Kamide et al., 1995; Aoki and Wada, 1996). Based on tissue distribution, ferredoxins that occur in plants can be classified into two broad categories. Photosynthetic ferredoxins have been shown to be light regulated and are predominantly expressed in photosynthetic tissues. In contrast, the heterotrophic ferredoxins have been shown to be independent of light regulation and have a more ubiquitous tissue distribution (Kimata and Hase, 1989; Hase et al., 1991a). Studies of ferredoxin and its influence on biochemical reactions have revealed that distinct isoforms have the potential to function differently in separate reactions. In tomato, the root-specific heterotrophic isoform (FdE) was found to have a cytochrome C (cyt C) reduction rate twice that found for the light-regulated photosynthetic isoforms (FdA and FdB) (Aoki and Wada, 1996). In addition, the maize (*Zea mays*) photosynthetic isoform (Fd I) was found to have a higher activity in assays of photoreduction of $NADP^+$ when compared with the heterotrophic isoform (Fd III). Conversely, when the Fd III isoform is compared with the Fd I isoform, the heterotrophic isoform was found to have a higher activity in assays of electron transfer from NADPH to ferredoxin (cyt C reduction). In addition, the activity of maize sulfite reductase was also shown to be higher when supplied with Fd III compared with Fd I when reducing electrons were supplied via NADPH/FNR (Yonekura-Sakakibara et al., 2000). However, maize Fd I and Fd III appeared to have no difference in activity in nitrite reductase assays (Hase et al., 1991b).

In our current study, we have characterized the activity of three unusual acyl-ACP desaturases (from coriander, *T. alata*, and geranium) as well as the activity of the ubiquitous Δ9 18:0-ACP desaturase (from spinach [*Spinacia oleracea*] and castor tissues) when supplied with ferredoxins from spinach, *Anabaena* sp., Arabidopsis, or impatiens (*Impatiens balsamina*). In addition, we have demonstrated that in tissues expressing both the Δ9 18:0-ACP and an unusual acyl-ACP desaturase, distinct ferredoxin isoforms can influence monoene production by differentially influencing the activity of the two acyl-ACP desaturases.

RESULTS

Characterization of Ferredoxin Isoforms

Two general types of ferredoxin, often referred to as photosynthetic and heterotrophic (or non-photosynthetic), are known to occur in higher plants (Wada et al., 1989; Morigasaki et al., 1990; Hase et al., 1991a; Kamide et al., 1995; Aoki and Wada, 1996; Aoki et al., 1998). However, the terms heterotrophic and photosynthetic are somewhat generic, describing tissue specificity and/or light regulation of ferredoxins. As a means to better classify each ferredoxin used in this study, we considered a combination of criteria (tissue source, light regulation, biochemical properties, and sequence). Characteristics of the four ferredoxin proteins examined in this study are summarized in Table I.

By virtue of light regulation and sequence similarity to other leaf ferredoxin isoforms (Somers et al., 1990; Bovy et al., 1995) the Arabidopsis ferredoxin (predominant leaf isoform) was tentatively classified as the photosynthetic type. Spinach ferredoxin was purified from mature leaves where the major leaf isoform constitutes at least 80% of the isolated ferredoxins and has been shown to be light regulated (Takahashi et al., 1981, 1983). Thus the purified spinach ferredoxin can be considered as the photosynthetic type. The impatiens ferredoxin clone has not been analyzed for tissue distribution or light regulation. However, this clone was isolated from non-photosynthetic impatiens tissue (white seed tissue), and thus is likely a heterotrophic type. The *Anabaena* sp. ferredoxin is from vegetative cells and has sometimes been referred to as a plant-type ferredoxin (Cheng et al., 1995; Navarro et al., 1995) based on the conservation of the 2Fe-2S cluster found in ferredox-

Table I. *Classification of ferredoxins from Anabaena, Arabidopsis, impatiens, and spinach*

Ferredoxin	Tissue[a]	Sequence Classification[b]	cyt C Reduction[c]
			nmol cyt C reduced min^{-1} (SE)
Arabidopsis	Leaf	Photosynthetic	3.1
Spinach	Leaf	Photosynthetic	3.6 (±0.42)
Anabaena sp.	Vegetative	Other[d]	7.5 (±0.7)
Impatiens	Seed	Heterotrophic	13.0 (±1.26)

[a] Tissue distribution is based on the tissue from which the ferredoxin was isolated. [b] Sequence classification was based on multiple sequence alignment (using DNAstar program) of 19 distinct ferredoxin protein sequences using the Clustal method. [c] cyt C reduction was measured when supplied with 2 nmol of ferredoxin in the presence of excess FNR and NADPH. cyt C reduction measurements represent the mean of multiple assays ($n = 5$, ± SE) in all cases except the Arabidopsis sample ($n = 1$). [d] This sequence could not be identified as photosynthetic or heterotrophic based on sequence alignments.

ins of many photosynthetic eukaryotes (Matsubara et al., 1980).

Verification of the initial ferredoxin classifications was provided by biochemical analysis as well as sequence alignments. Photosynthetic ferredoxins are more efficient at donating electrons to $NADP^+$ (via FNR), whereas heterotrophic ferredoxins are more efficient at accepting electrons from NADPH (via FNR) (Hase et al., 1991b; Aoki and Wada, 1996). Thus, a convenient method to analyze ferredoxin electron transfer, and thus classify as photosynthetic or heterotrophic, has been cyt C reduction assays. cyt C reduction analysis of the four ferredoxin isoforms indicated marked differences between samples (Table I). The activities of the photosynthetic ferredoxin from spinach (3.6 nmol cyt C reduced min^{-1}) and from Arabidopsis (3.1 nmol cyt C reduced min^{-1}) were not significantly different. In contrast, the *Anabaena* sp. and impatiens ferredoxins had cyt C reduction rates approximately 2- and 4-fold higher, respectively, than either photosynthetic ferredoxin. Thus, cyt C reduction assays indicated that the heterotrophic impatiens ferredoxin supported the highest cyt C reduction activity with both photosynthetic ferredoxins from spinach and Arabidopsis supporting the lowest cyt C reduction activity.

In addition to tissue source and biochemical activities of ferredoxins, sequence analysis has been useful in classification of ferredoxins as photosynthetic or heterotrophic (Wada et al., 1989; Hase et al., 1991a; Alonso et al., 1995). As shown in Figure 1, multiple sequence alignments (by clustal analysis) of ferredoxin sequences available in the GenBank database indicated the spinach and Arabidopsis ferredoxins examined in this study grouped with other photosynthetic ferredoxins whereas the impatiens ferredoxin grouped with heterotrophic ferredoxins. *Anabaena* sp. ferredoxin did not group directly with either type of plant ferredoxin but is more similar to the heterotrophic group. Thus, all lines of evidence (cyt C reduction assays, sequence alignment, tissue distribution, and light regulation) support the classification of spinach and Arabidopsis ferredoxins as photosynthetic, impatiens ferredoxin as heterotrophic, and *Anabaena* sp. ferredoxin as distinct from either type.

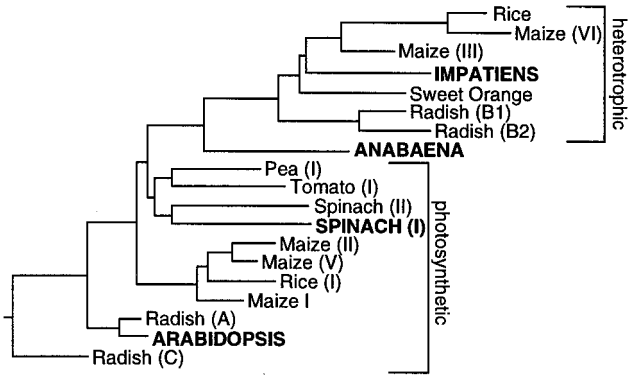

Figure 1. Relationship of photosynthetic and heterotrophic ferredoxin proteins sequences. Amino acid sequences from ferredoxins classified as photosynthetic or heterotrophic (based on tissue distribution, light regulation, and/or biochemical activity) as well as ferredoxin from vegetative cells of *Anabaena* sp. were aligned using the clustal method (DNAstar program). Isoforms from the same species are differentiated by roman numerals or letters in parentheses. GenBank accession numbers for the sequences used in this analysis are as follows: *Anabaena* sp. (P06543), Arabidopsis (P16972), sweet orange (S62722), impatiens (AF233452), tomato I (Q43517), rice (BAA 06456), rice I (P11051), Pea (P09911), radish A (JX0082), radish B1 (P14936), radish B2 (P14937), radish C (AAB33406), spinach I (P00221), spinach II (P00224), maize I (P27787), maize II (BAA32348), maize III (P27788), maize V (P27789), and maize VI (P94044). Ferredoxins used in this study are denoted in bold capital letters.

Unusual Acyl-ACP Desaturases Are Most Active with Heterotrophic Ferredoxin

In this study, we have examined the activity of three distinct acyl-ACP desaturase systems from different plant families (*T. alata* Δ6 16:0-ACP desaturase, coriander Δ4 16:0-ACP desaturase, and geranium Δ9 14:0-ACP desaturase) with the ferredoxins described above. To determine the influence of ferredoxin type on unusual acyl-ACP desaturase activity, the Δ6 16:0-ACP desaturase of *T. alata* was assayed in vitro under conditions where the reaction rate was linear with respect to both enzyme and ferredoxin concentrations. As shown in Figure 2A, activity of the *T. alata* desaturase was strongly influenced by the type of ferredoxin cofactor at all concentrations tested. When the Δ6 16:0-ACP desaturase was supplied with 1 μM of either spinach or Arabidopsis photosynthetic ferredoxin, no activity could be detected. In contrast, desaturase activity was easily measured at levels as low as 0.1 μM with either *Anabaena* sp. or impatiens ferredoxin. Furthermore, activity of the Δ6 16:0-ACP desaturase, when supplied with 0.1 μM *Anabaena* sp. or impatiens ferredoxin, was still higher than when supplied with 10 μM spinach or Arabidopsis ferredoxin. Thus, it is apparent that the photosynthetic ferredoxins support only a small fraction of activity found with heterotrophic or cyanobacterial ferredoxins (compare 10 μM spinach and Arabidopsis ferredoxin to 0.1 μM *Anabaena* sp. or impatiens ferredoxin).

As shown in Figure 2, B and C, the strong effect of ferredoxin isoforms on the unusual acyl-ACP desaturase activities of coriander and geranium was similar. With the coriander Δ4 16:0-ACP desaturase (Fig. 2B), *Anabaena* sp. ferredoxin supported an activity that was at least 18-fold higher than the spinach ferredoxin. With geranium trichome protein extracts (Fig. 2C), both *Anabaena* sp. and impatiens ferredoxin provide similar high desaturase activity, whereas no activity could be detected using spinach ferredoxin as

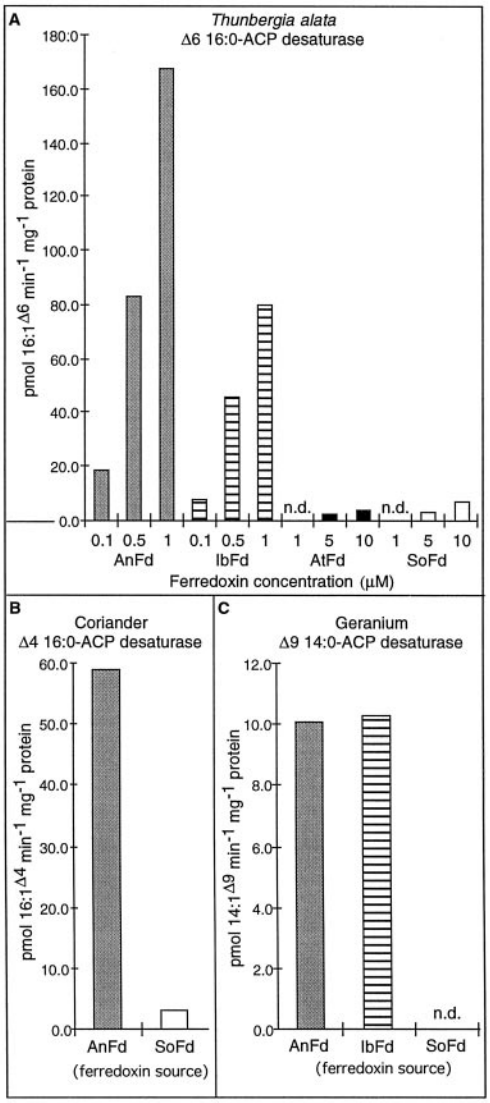

Figure 2. Influence of ferredoxin type on in vitro acyl-ACP desaturase activity. A, Δ6 16:0-ACP desaturase activity in *T. alata* endosperm. Each sample was supplied with concentration of ferredoxin indicated and 0.02 mg of crude protein extract. The *T. Alata* assays with *Anabaena* sp. (AnFd) or impatiens (IbFd) ferredoxin were 15 min, whereas the assays with Arabidopsis (AtFd) or spinach (SoFd) ferredoxin were 40 min. B, Δ4 16:0-ACP desaturase activity in coriander endosperm. Assays were supplemented with 40 μM of ferredoxin and 0.2 mg of crude protein extract and reactions were terminated at 7 min. C, Δ9 14:0-ACP desaturase activity in geranium trichomes. The geranium desaturase assays contained 5 μM of ferredoxin and 0.1 mg of crude protein extract and were terminated at 15 min. n.d., Samples in which no desaturase activity could be detected.

the electron donor. Thus, in all cases tested, the impatiens and *Anabaena* sp. ferredoxin supported higher desaturase activity than did either photosynthetic ferredoxin (Fig. 2). Based on these and additional data (not shown) we estimate that the heterotrophic ferredoxins are at least 10- to 20-fold more effective cofactors than either photosynthetic ferredoxin.

Do Ferredoxins Influence Stearoyl-ACP Desaturases to the Same Extent as the Unusual Acyl-ACP Desaturases?

We also examined whether the source of ferredoxin has a similar strong influence on the Δ9 18:0-ACP desaturase activity in leaves of spinach and castor as well as developing endosperm of castor. Figure 3A demonstrates the response of spinach leaf Δ9 18:0-ACP desaturase activity to varying concentrations of spinach or impatiens ferredoxin. Consistent with assays of the unusual acyl-ACP desaturases, the heterotrophic ferredoxin was capable of supporting a higher activity and at a lower concentration than the photosynthetic ferredoxin (compare 0.08 μM samples). Unlike the unusual acyl-ACP desaturases, the Δ9 18:0-ACP desaturase occurs in both leaf and seed tissues. To determine if the tissue origin of the desaturase influences which ferredoxin isoform is preferred, desaturase activity from castor developing seed endosperm was compared with desaturase activity from

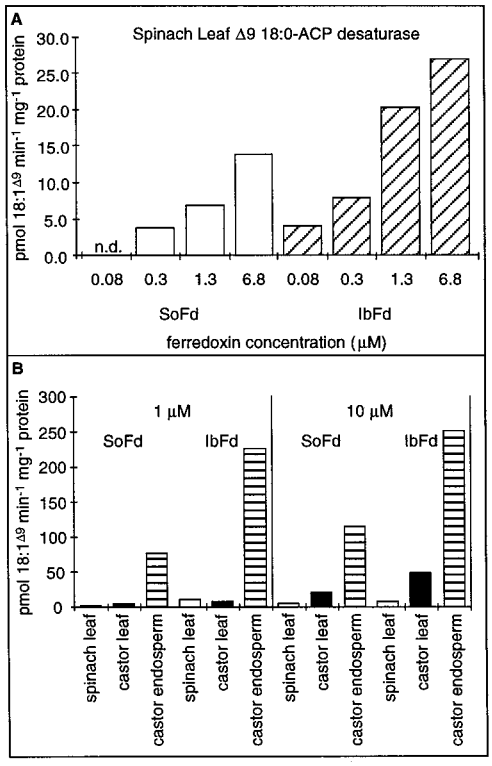

Figure 3. Influence of ferredoxin source on Δ9 18:0-ACP desaturase activity. A, Activity of the spinach leaf Δ9 18:0-ACP desaturase was analyzed in crude leaf protein extracts (0.05 mg) supplied with spinach (SoFd) or impatiens (IbFd) ferredoxins at noted concentrations. Reactions were terminated after 30 min. B, The influence of spinach and impatiens ferredoxin on activity of Δ9 18:0-ACP desaturases from spinach and castor leaves was compared with the activity from castor-developing endosperm. Crude protein extracts of spinach leaf (0.05 mg), castor leaf (0.025 mg), or castor-developing endosperm (0.01 mg) were supplied with 1 or 10 μM ferredoxin. Assays containing 10 μM ferredoxin were terminated at 15 min, whereas assays containing 1 μM ferredoxin were terminated at 30 min.

leaves (Fig. 3B). Consistent with previous assays, the heterotrophic ferredoxin supported higher levels of activity in all tissues when compared with the photosynthetic spinach ferredoxin. The heterotrophic ferredoxin consistently supported 2- to 3-fold higher Δ9 18:0-ACP desaturase activity when compared with the photosynthetic spinach ferredoxin regardless of tissue source or species. This is in contrast to the unusual acyl-ACP desaturases where impatiens and *Anabaena* sp. ferredoxin influence activity to a different extent between species. This could indicate a more general role for the heterotrophic ferredoxin in Δ9 18:0-ACP desaturase activity and/or the existence of more highly specific heterotrophic ferredoxins in the endogenous species where the unusual acyl-ACP desaturases are found.

Heterotrophic Ferredoxin Alters the Relative Activity of the Unusual Acyl-ACP Desaturase and Δ9 18:0-ACP Desaturase

An initial indication that ferredoxin differentially influences the activity of the unusual acyl-ACP desaturases compared with the Δ9 18:0-ACP desaturase was found in studies of petroselinic acid biosynthesis in coriander. Cahoon and Ohlrogge (1994) reported that addition of spinach ferredoxin to coriander extracts stimulated the incorporation of [1-^{14}C]malonyl-coenzyme A (CoA) into oleic acid by 2-fold but had no significant influence on the activity of the Δ4 16:0-ACP desaturase (production of petroselenic acid remained constant). Furthermore, in *T. alata* Cahoon et al. (1994) found that the activity of the Δ9 18:0-ACP desaturase was 1.7-fold higher than the Δ6 16:0-ACP desaturase in assays supplied with spinach ferredoxin. This result was unexpected, as 16:1$^{\Delta 6}$ comprised more than 85% of the total fatty acids in *T. alata* endosperm (Spencer et al., 1971).

To compare the unusual acyl-ACP desaturase to stearoyl-ACP desaturase from the same species and tissue under identical assay conditions we have examined developing *T. alata* seeds. During seed development, we can consider oilseed tissue to have three developmental periods in respect to lipid biosynthesis. Early in development, tissue would be primarily involved in membrane lipid biosynthesis, whereas in late development the tissue would be primarily involved in storage lipid biosynthesis. At some point during development, a transitional period between membrane and storage lipid biosynthesis would occur. To better define the developmental profile of *T. alata* lipid biosynthesis, we analyzed the activity of the Δ6 16:0-ACP and Δ9 18:0-ACP desaturases as well as lipid deposition during endosperm development.

Lipid deposition was examined between 10 and 35 d after pollination (DAP) and as shown in Figure 4A, lipid deposition was in the linear range between 10 to 16 DAP. The profile of Δ6 16:0-ACP and Δ9 18:0-ACP acyl-ACP desaturase activities during development

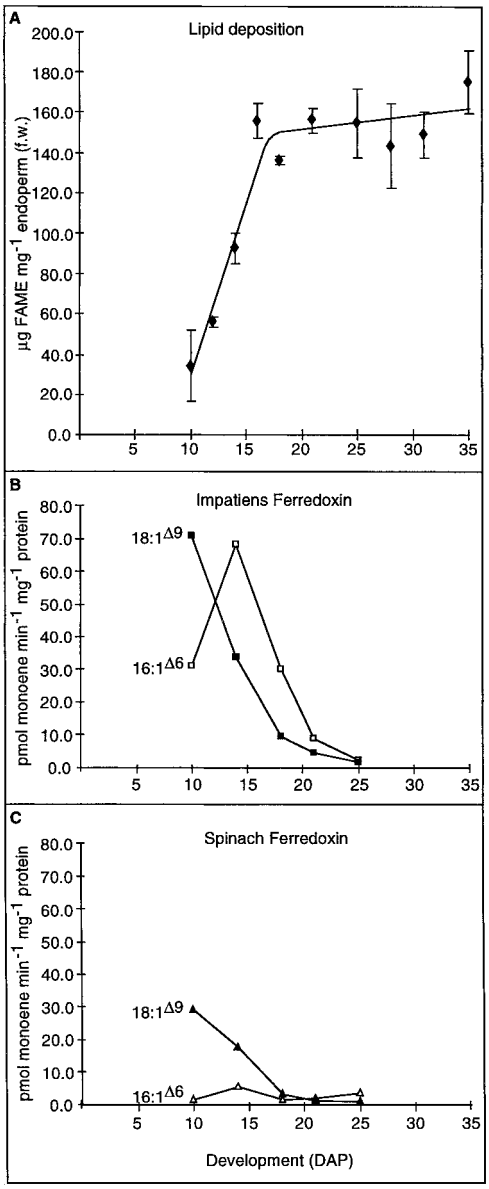

Figure 4. Developmental analysis of *T. alata* lipid production and acyl-ACP desaturase activity. A, Lipid deposition in developing seed endosperm of *T. alata*. Replicate samples ($n = 3$) were harvested and extracted for each developmental time point. Results are expressed as μg fatty acid methyl ester (FAME) mg^{-1} fresh weight (f.w.) endosperm. Error bars represent SE. Developmental profiles of acyl-ACP desaturase activities in *T. alata* developing endosperm were analyzed when supplied with impatiens ferredoxin (B) or spinach ferredoxin (C). Desaturase assays contained 10 μM ferredoxin (either spinach or impatiens) and 0.02 mg of crude protein extract. All reaction were terminated at 15 min. White symbols, Activities of the Δ6 16:0-ACP desaturase. Black symbols, Activities of the Δ9 18:0-ACP desaturase. In all developmental studies, flowers were hand pollinated, tagged, and harvested at specified DAP.

was determined with assays supplemented with either heterotrophic (Fig. 4B) or photosynthetic (Fig. 4C) ferredoxin. When assayed with impatiens ferredoxin, the Δ9 18:0-ACP desaturase activity predominates at

the earlier stage of development (10 DAP). Two days later, the pattern has reversed with the Δ6 16:0-ACP desaturase activity exceeding Δ9 18:0-ACP desaturase activity. At 18 d, Δ6 16:0-ACP desaturase activity was 3-fold higher than the Δ9 18:0-ACP desaturase activity. Beyond 18 d, activities of Δ6 and Δ9 acyl-ACP desaturases, whether supplied with spinach or impatiens ferredoxin, declined and were not readily distinguishable. Thus, at 10 DAP, *T. alata* seed development likely is in transition from a tissue that is predominantly synthesizing membrane lipids to a tissue that is predominantly synthesizing storage lipids. The activity of both desaturase enzymes was substantially lower when assayed with spinach ferredoxin, and furthermore, the relationship between the two activities was altered such that the Δ9 18:0-ACP desaturase activity exceeded the Δ6 16:0-ACP desaturase at almost all times when supplied with the spinach ferredoxin.

More detailed analyses of the relative rates of the two desaturases were performed on mid-stage *T. alata* endosperm (16 DAP). As indicated in Figure 5, comparisons of activities in the linear range for each reaction indicated that when supplemented with impatiens ferredoxin, the Δ6 16:0-ACP desaturase activity (255 pmol $16{:}1^{\Delta 6}$ min^{-1} mg^{-1}) was 1.8-fold higher than the Δ9 18:0-ACP desaturase activity (145 pmol $18{:}1^{\Delta 9}$ min^{-1} mg^{-1}). In contrast, in assays supplemented with spinach ferredoxin, the Δ9 18:0-ACP desaturase activity (97.6 pmol $18{:}1^{\Delta 9}$ min^{-1} mg^{-1}) was 1.7-fold higher than the Δ6 16:0-ACP desaturase activity (57.4 pmol $16{:}1^{\Delta 6}$ min^{-1} mg^{-1}). Although both enzymes have higher activity with impatiens than with spinach ferredoxin, the stimulation is 4.5-fold for the Δ6 16:0-ACP desaturase whereas it is only 1.5-fold for the Δ9 18:0-ACP desaturase. In addition, we found that the *Anabaena* sp. ferredoxin had an even greater influence than the heterotrophic ferredoxin on the relative acyl-ACP desaturase activities in *T. alata* (data not shown).

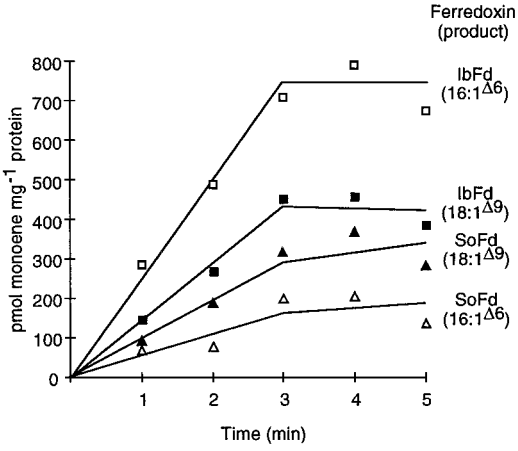

Figure 5. Ferredoxin type alters products of acyl-ACP desaturase assays in *T. alata* endosperm. Flowers were hand pollinated, and seeds were harvested and dissected at 16 DAP. Assays contained 0.02 mg of crude protein extract and 10 μM ferredoxin. Reaction were terminated at noted times. Square symbols, Assays supplied with impatiens ferredoxin; triangle symbols, assays supplied with spinach ferredoxin. White symbols, Activity of the Δ6 16:0-ACP desaturase; black symbols, activity of the Δ9 18:0-ACP desaturase.

Additional evidence for changes in relative activities of the ubiquitous and unusual acyl-ACP desaturases was found in [1-^{14}C]malonyl-CoA labeling studies in coriander. When coriander is supplied with [1-^{14}C]malonyl-CoA in the presence of spinach ferredoxin, the majority product is saturated fatty acids (43%). When supplied with [1-^{14}C]malonyl-CoA in the presence of *Anabaena* sp. ferredoxin, saturated fatty acids account for only 15% of the labeled fatty acids. Thus, the *Anabaena* sp. ferredoxin supported higher levels of total acyl-ACP desaturase activity. Although *Anabaena* sp. ferredoxin appears to increase activity of both acyl-ACP desaturases in coriander endosperm, upon analysis of monoene composition, the largest increase is found in $18{:}1^{\Delta 6}$ (elongation product from the Δ4 16:0-ACP desaturase) (data not shown).

DISCUSSION

After the discovery of the acyl-ACP desaturase reaction and its preference for ferredoxin as electron donor, essentially all studies of acyl-ACP desaturases in plants have used spinach ferredoxin as the cofactor for in vitro studies. Ferredoxin is a relatively abundant and easily purified protein from green material such as spinach leaves. Recently the level of the major leaf isoform (Fd I) was estimated at 37 μM, whereas the heterotrophic isoform (Fd III) was less than 0.4 μM in maize leaves (Yonekura-Sakakibara et al., 2000). Because the acyl-ACP desaturase has been easily assayed in the presence of spinach ferredoxin, one assumption that has arisen from these studies is that the abundant leaf ferredoxin associated with electron transfer from photosystem I to $NADP^+$ is involved in vivo in electron transfer to the desaturase. The results of the study described here, together with the recognition that plant tissues express several ferredoxin isoforms, raises the question of whether this assumption is correct or whether the acyl-ACP desaturase reaction may be associated with specific isoforms of ferredoxin specialized for this reaction. The heterotrophic ferredoxins are much less abundant than photosynthetic isoforms. Based on the analysis of relative ferredoxin isoform abundance during tomato fruit development (Aoki and Wada, 1996), heterotrophic ferredoxin is estimated to be at least 20-fold less abundant compared with photosynthetic ferredoxins. Based on the estimated maize leaf photosynthetic ferredoxin (Fd I, 37 μM) and heterotrophic ferredoxin levels (Fd III, 0.4 μM) the relative abundance (on a protein basis) of the heterotrophic ferredoxins (in roots and leaves) (Yonekura-Sakakibara et al., 2000) can be estimated to be between 0.4 to 5

μM. We also analyzed the levels of photosynthetic and heterotrophic ferredoxin expressed sequence tags (ESTs) in the Arabidopsis EST database (GenBank release, version 117.0) and have found 55 photosynthetic ferredoxin ESTs and two heterotrophic ferredoxin ESTs. Furthermore, two additional Arabidopsis ferredoxin isoforms not found in the EST database (one heterotrophic and one photosynthetic) are present as genomic sequences available in GenBank. The Δ9 18:0-ACP desaturase and 18:0-ACP levels were recently estimated at up to 0.4 μM (Mekhedov et al., 2000). However, a more direct comparison may be made using the abundance of EST clones in the Arabidopsis EST database. Mekhedov et al. (2000) found four Δ9 18:0-ACP desaturase ESTs, whereas two heterotrophic ferredoxin ESTs are found in the database. Therefore, based on estimates of protein levels and EST abundance it is likely that at least in some tissues, the electron donating cofactor for acyl-ACP desaturases is at a concentration similar to the desaturase enzyme. This consideration lends support to the hypothesis that the components of the desaturase and perhaps of the fatty acid synthase pathway occur together in some type of supramolecular structure.

The results presented in this study help to explain two previously puzzling observations. First, Cahoon et al. (1994) found that addition of spinach ferredoxin to coriander extracts stimulated production of oleic acid, but not petroselinic acid. Second, in vitro assays of the *T. alata* Δ6 16:0-ACP desaturase indicated activities 1.7-fold lower compared with Δ9 18:0-ACP desaturase activity (Cahoon et al., 1994). In light of the results shown in Figure 2, it seems probable that the low in vitro activity of unusual acyl-ACP desaturases is at least partly due to the absence of an appropriate native ferredoxin. Our results indicate that the activity of these desaturases can be increased substantially when assayed with heterotrophic or *Anabaena* sp. ferredoxins. In this study we have only tested ferredoxins from heterologous sources, thus it remains a distinct possibility that ferredoxin isoforms exist in *T. alata*, coriander, or geranium that have specific kinetic interaction with the acyl-ACP desaturases, which would result in even higher activities than those obtained with impatiens or *Anabaena* sp. ferredoxin.

The results presented in this study are related to work over 30 years ago by Nagai and Bloch (1966, 1968) in which a comparison was made between spinach and Euglena ferredoxins. In the presence of Euglena ferredoxin, the Euglena stearoyl-ACP desaturase was 10-fold more active than when supplied with the spinach ferredoxin. Furthermore, the spinach stearoyl-ACP desaturase was also found to be more active with Euglena ferredoxin than with spinach ferredoxin.

Consistent with reports that several biochemical reactions were influenced based on the altered ability of ferredoxin types to either accept or donate electrons (Hase et al., 1991b; Aoki and Wada, 1996), the photosynthetic sources of ferredoxin we tested supported the lowest level of activity in assays where ferredoxin donates electrons via NADPH (cyt C reduction and acyl-ACP desaturase reactions). In contrast, the heterotrophic ferredoxin we tested supported higher cyt C reduction activity and acyl-ACP desaturase activity. It is important to note that the heterotrophic ferredoxin supported higher cyt C reduction than the *Anabaena* sp. ferredoxin, whereas the *Anabaena* sp. ferredoxin supported greater or equal levels of acyl-ACP desaturase activity when compared with impatiens heterotrophic ferredoxin. These results indicate two things. First, the difference in activity is not likely due to differences in redox potential. Second, interactions with FNR or impurities in the ferredoxin preparation are not likely influencing these reactions. Redox potential of *Anabaena* sp. ferredoxin has been determined to be −440 mV (Hurley et al., 1993), whereas the major leaf isoform (Fd I) in spinach has a redox potential measured at −400 mV (Aliverti et al., 1995; Piubelli et al., 1996). Since NADPH/$NADP^+$ redox potential is −320 mV the transfer of electrons from NADPH to the *Anabaena* sp. ferredoxin is less favored compared with spinach Fd I. Despite a less favorable redox potential in terms of electron flow from NADPH, the *Anabaena* sp. ferredoxin supported higher levels of activity in acyl-ACP desaturase and cyt C reduction assays, both of which required electron transfer from NADPH. In addition, ferredoxin interactions with FNR are not likely influencing the desaturase reactions. Using maize, interactions of heterotrophic and photosynthetic ferredoxins with FNR (leaf or root type) were shown to differentially influence sulfite reductase activity when supplied with reductant through NADPH (Yonekura-Sakakibara et al., 2000). However, acyl-ACP desaturase assays supplied with *Anabaena* sp. ferredoxin, NADPH and either spinach leaf or maize root FNR showed no difference in activity (data not shown). If the differences in acyl-ACP desaturase reactions were the result of redox potential or interaction between ferredoxin and FNR, the relative order of ferredoxin supported activity would have likely been the same with the different acyl-ACP desaturases. However, in assays of two distinct acyl-ACP desaturases in the same tissue, ferredoxin source influenced each desaturase to a different extent. Thus, the simplest interpretation of these data is that acyl-ACP desaturase activity is influenced by direct interactions with the specific ferredoxins rather than by differences in redox potential or ferredoxin interactions with FNR.

Evidence is now accumulating that ferredoxin interactions with its electron partners are dictated at least in part by electrostatic interactions. Investigation of maize ferredoxin III has shown that the binding site for individual enzymes is differentially influ-

enced by specific mutations and thus the binding site for each protein is not identical (Akashi et al., 1999). In addition, Walker et al. (1991) analyzed the interactions of ferredoxin with FNR in both *Anabaena* sp. strain PCC 7119 and spinach and found that electrostatic interactions most likely contributed to the formation of protein complex that permits electron transfer. Furthermore, it has been determined that alterations at specific positions (E92) in spinach ferredoxin could increase activity of ferredoxin in supporting NADPH/FNR mediated cyt C reduction and reduced the activity of ferredoxin in supporting photoreduction of $NADP^+$ by photosystem I (Piubelli et al., 1996). These studies collectively show it is possible to alter kinetic activity without significantly altering redox potential.

One long-term application of the unusual acyl-ACP desaturases may be to produce specialty fatty acids in transgenic oil crops. We have recently identified specialized ACP isoforms as one potential component for optimal monoene production (Suh et al., 1999), and results of our current study suggest ferredoxin might also be important to monoene production. The critical evaluation of the ability of ACP and ferredoxin to support higher acyl-ACP desaturase activity will be co-expression of both cofactors together with the unusual acyl-ACP desaturases in transgenic plants. The production of monoenes in transgenic oilseeds has now become a surprisingly complex undertaking. To date, three additional components (acyl carrier protein, β-ketoacyl-acyl carrier protein synthase, and thioesterase) to the acyl-ACP desaturase and ferredoxin have been identified that may be necessary for production in transgenic oilseeds (Dörmann et al., 1994; Mekhedov et al., 1997; Suh et al., 1999). With the realization of the complexity of these limitations, it is attractive to now speculate on the possibility of two potentially overlapping fatty acid biosynthetic pathways in the seed of *T. alata* and coriander and trichomes of geranium. One system would function in the production of membrane lipids, the second would function in the production of storage lipids. If such a dual system exists, some of the components (e.g. acetyl coenzyme A and β-ketoacyl-ACP synthase) are likely shared between the systems. However, we would envision that in such a system, targeting of acyl groups to either membrane lipid production or storage lipid production could be dictated by ACP isoforms. In addition, the interaction of ACP isoforms with other components (for example ferredoxin, β-ketoacyl-ACP synthase, or thioesterase) may have an additive influence on monoene production.

MATERIALS AND METHODS

Plant Material

Thunbergia alata, coriander (*Coriandrum sativum*), geranium (*Pelargonium* × *hortorum*), and castor (*Ricinus communis*) were grown under standard greenhouse conditions. Spinach (*Spinacia oleracea*) was grown in growth chambers (8-h-light period, 23°C). Assays were conducted with freshly dissected tissue. Developing seeds were harvested directly onto ice, then the endosperm was dissected from the seed coat and embryo. Geranium trichomes were harvested from flower pedicles as previously described (Yerger et al., 1992). Trichome material was used immediately after harvest, however as part of the trichome isolation method, the tissue was frozen in liquid nitrogen prior to trichome isolation. For studies involving distinct developmental stages of *T. alata* endosperm, flowers were hand pollinated and tagged. Tissue was then harvested at specific DAP.

Purification of Ferredoxin and FNR

Spinach ferredoxin, purified from leaves, was purchased (Sigma, St. Louis). The Arabidopsis (Arabidopsis Resource Center, EST no. 305C11T7), impatiens (*Impatiens balsamina*, kindly provided by Dr. Edgar Cahoon, DuPont, Willmington, DE), and *Anabaena* sp. 7120 ferredoxins (Cheng et al., 1995) were cloned into pET expression vectors (Novagen, Madison, WI), overexpressed in *Escherichia coli* [BL21 (DE3)/pLysS], then purified based on procedures outlined by Cheng et al. (1995). Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.4 mM in 2-L cultures. Cells were pelleted by centrifugation at 5,000*g* then frozen at −20°C until purification. Cells were thawed on ice in 50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl (pH 7.5), then lysed using a French pressure unit at 20,000 psi (138 MPa). To ensure complete lysis, the crude extract was incubated at 30°C for 20 min with lysozyme (0.1 mg/mL) and DNase (0.05 mg/mL). Insoluble materials were pelleted by centrifugation for 15 min, 4°C, at 10,000*g* and the supernatant was then applied to a DEAE-cellulose column equilibrated with 50 mM Tris-HCl (pH 7.5). Protein fractions were eluted from the column with a linear gradient of 50 mM Tris-HCl (pH 7.5) to 0.5 M NaCl in 50 mM Tris-HCl (pH 7.5). Pooled fractions containing ferredoxin were concentrated and desalted in 50 mM Tris-HCl (pH 7.5) using an ultrafree centrifugal filter (Biomax-5K nominal molecular weight limit membrane, Millipore, Bedford, MA), then further purified by gel filtration using superdex 75 column (Pharmacia Biotech, Piscataway, NY). Fractions containing ferredoxin were again pooled and concentrated as above. Final purification was achieved using an ion-exchange column (protein pak Q 8HR, Waters, Milford, MA). Samples were again pooled and concentrated as above. Concentration of ferredoxin was determined using the extinction coefficient 10.4 mM^{-1} cm^{-1} at λ_{422} as previously reported (Wada et al., 1974).

FNR was produced by overexpression of the maize (*Zea mays*) root FNR clone (Ritchie et al., 1994) as described above. Initial purification of FNR was as described for ferredoxin except 25 mM Tris-HCl (pH 7.5) was used. The FNR extract was applied to a DEAE-cellulose column equilibrated with 25 mM Tris-HCl (pH 7.5) then eluted and

collected in 1.5-mL fractions with a linear gradient of 25 mM Tris-HCl (pH 7.5) to 0.3 M NaCl in 25 mM Tris-HCl (pH 7.5). All fractions were assayed for FNR by cyt C reduction assays (see below). The results of these assays indicated all FNR activity was found in the column flow through and column rinse. The fractions were pooled and concentrated using an ultrafree centrifugal device (Biomax-30 K NMWL membrane). The sample was further purified by gel filtration (S-300 Hi-load 16 column, Pharmacia Biotech). Fractions containing FNR were concentrated as above, then tested for FNR activity by cyt C reduction assays. The specific activity of the purified FNR was determined to be 10 units/mL (1unit = 1 μmol cyt C reduced min^{-1} mg^{-1} protein).

cyt C Reduction Assay

cyt C reduction assays were based on Shin (1971). Reactions were carried out in 1-mL volumes containing 80 μM cyt C (bovine heart, Sigma), 50 mM Tris-HCl, pH 7.6, and 100 μM NADPH. Assays used to determine the activity of ferredoxin isoforms contained 2 μM ferredoxin and 0.59 unit/mL FNR. Assays used to determine FNR activity contained 2 μM impatiens ferredoxin. Reactions were started by addition of cyt C to the samples and spectrophotometer readings were taken at λ_{550} for 1,500 s, at 30-s intervals. Change in absorbency over time was used to calculate the linear range of reactions for specific activity calculations using the extinction coefficient 19.1 mM^{-1} cm^{-1} (Nakamura and Kimura, 1971).

T. alata Endosperm Lipid Deposition

Endosperm tissue was harvested at time points between 10 and 35 DAP. Three replicates were harvested at each time point. Tissue was weighed and internal standard (1,2,3-triheptadecanoylglycerol, Sigma) was added. Total lipids were extracted using the method of Bligh and Dyer (1959). After extraction, the sample was evaporated to dryness under a steady stream of nitrogen gas. Samples were trans-methylated in a 1-mL solution containing 10% (w/v) BCl_3 in methanol (Sigma) and 30% (v/v) toluene as described (Morrison and Smith, 1964). Fatty acid methyl esters were analyzed by gas chromatography using a CP-Sil 88 capillary column (50-m × 0.25-mm i.d., Chrompack, Middelburg, The Netherlands) with the oven temperature programmed from 150°C to 200°C at 2°C/min.

Acyl-ACP Desaturase Assays

[1-^{14}C]Acyl-ACP substrates were prepared using the procedure of Rock and Garwin (1979) with either ACP from *E. coli* (substrates for *T. alata* and geranium assays) or coriander ACP (Suh et al., 1999) overexpressed in *E. coli* (substrates for coriander assays). All labeled fatty acids used for the synthesis of acyl-ACP had specific activities of 57 Ci/mol (American Radiolabeled Chemicals, St. Louis). [1-^{14}C]Malonyl-CoA was synthesized from [1-^{14}C]acetate (45 Ci/mol, American Radiolabeled Chemicals) as described (Roughan, 1994).

Crude protein homogenates of endosperm (*T. alata*, coriander, and castor), trichomes (geranium), or leaves (spinach or castor) were used for acyl-ACP desaturase assays. All tissues except coriander endosperm were homogenized in extraction buffer containing 0.1 M Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 5 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM KCl, 1 mM ascorbate, 0.1% (w/v) bovine serum albumin, 1% (w/v) polyvinylpyrrolidone, and 2% (w/v) polyvinylpolypyrrolidone. In these extractions, 5 μL of extraction buffer per milligram of tissue was used. It was not possible to measure trichome tissue quantity, so a minimal volume of buffer was added to the purified trichomes, then transferred to a 1.5-mL microcentrifuge tube. The trichome sample was vortexed extensively, then tissue was pelleted by a brief centrifugation, then homogenized using a micropestle. After homogenization, the supernatant from each sample was clarified by two centrifugations at approximately 3,000*g*, 15 min at 4°C. Crude protein homogenate of coriander tissue was made using the same buffer as above, except the buffer contained 2 mM dithiothreitol, and did not contain polyvinylpyrrolidone or polyvinylpolypyrrolidone. After homogenization with a glass homogenizer, the coriander homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA). Protein quantity was determined from the supernatant using the Bradford assay (Bradford, 1976) with protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA).

Acyl-ACP desaturase assays of *T. alata* and castor endosperm, geranium trichomes, and spinach and castor leaves were conducted as previously reported (Cahoon et al., 1994) with the following modifications. Assays contained 0.2 mg/mL bovine serum albumin, 10 μM ferredoxin (unless otherwise noted), maize root FNR was used instead of spinach FNR, and 124-pmol substrate (14:0-ACP, 16:0-ACP, or 18:0-ACP) was supplied. Assays of *T. alata* tissue contained 0.02 mg of crude protein extract, assays of geranium trichomes contained 0.1 mg of crude protein extract, assays of spinach leaf contained 0.05 mg of crude protein extract, assays of castor leaf contained 0.025 mg of crude protein extracts, and assays of castor endosperm contained 0.01 mg of crude protein extracts. Assays were incubated at room temperature (21°C) with shaking (100 rpm) for noted times. Reactions were terminated and saponified as described (Cahoon et al., 1994). Fatty acids were trans-methylated as described above.

Acyl-ACP desaturase assays in coriander contained 2 mM ascorbate, 0.75 mM NADPH, 1 mM NADH (NADPH and NADH were prepared from fresh stocks in 0.1 M Tricine {*N*-[tris(hydroxymethyl)methyl]Gly}, pH 8.0), 800 units of catalase, 2 mM ATP, 32 mM PIPES (1,4-piperazinediethanesulfonic acid) (pH 6.0), 0.04 mM ferredoxin, 80 milliunits of FNR (spinach FNR, Sigma), 124 pmol of substrate, and 0.2 mg of crude protein extract in a total reaction volume of 0.25 mL. [1-^{14}C]Malonyl-CoA feeding studies of acyl-ACP desaturase activity in coriander were conducted under identical reactions conditions described for coriander acyl-ACP desaturase assays, except

2.7 nmol of [1-^{14}C]malonyl-CoA (350,000 dpm) was added in place of [1-^{14}C]16:0-ACP. Reactions were incubated at room temperature (21°C) with shaking (100 rpm) for 7 min. Coriander desaturase reactions were terminated by the addition of 0.04 mL of glacial acetic acid and 4.5 mL of acetone followed by evaporation to complete dryness under a steady steam of nitrogen gas. Fatty acids were converted to methyl esters by heating in 1 mL of 3 N methanolic-HCl at 90°C for 35 min.

Desaturase reaction products were analyzed by argentation thin-layer chromatography (TLC) as described (Morris, 1966) with the following exceptions. Silica plates (K6 60A, Whatman, Clifton, NJ), were treated with a solution of 15% (w/v) $AgNO_3$ in acetonitrile for 15 min. Samples were spotted and the plates developed sequentially to 5.5, 11, and 16.5 cm in toluene at −20°C. Radioactive products were identified and quantified using either an Instant Imager (Packard Instruments, Downers Grove, IL) or Phosphorimager (Molecular Dynamics, Sunnyvale, CA), or by liquid scintillation counting of TLC scrapings. Since methyl esters of 16:1$^{\Delta 4}$ and 18:1$^{\Delta 9}$ migrate very closely in this TLC system, monounsaturated methyl esters in coriander samples were eluted from TLC plate scrapings with hexane/ethyl ether (2:1, v/v). The monounsaturated methyl esters were then cleaved at the double bond using permanganate-periodate oxidation (Christie, 1982). Chain lengths of oxidation products were determined relative to ^{14}C fatty acid standard by reverse-phase TLC (KC18 silica gel, 250 μM layer, Whatman).

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