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Supply of Fatty Acid Is One Limiting Factor in the Accumulation of Triacylglycerol in Developing Embryos[1]

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The metabolic factors that determine oil yield in seeds are still not well understood. To begin to examine the limits on triacylglycerol (TAG) production, developing *Cuphea lanceolata*, *Ulmus carpinifolia*, and *Ulmus parvifolia* embryos were incubated with factors whose availability might limit oil accumulation. The addition of glycerol or sucrose did not significantly influence the rate of TAG synthesis. However, the rate of ^{14}C-TAG synthesis upon addition of 2.1 mM ^{14}C-decanoic acid (10:0) was approximately four times higher than the in vivo rate of TAG accumulation in *C. lanceolata* and two times higher than the in vivo rate in *U. carpinifolia* and *U. parvifolia*. In *C. lanceolata* embryos, the highest rate of ^{14}C-TAG synthesis (14.3 nmol h^{-1} embryo^{-1}) was achieved with the addition of 3.6 mM decanoic acid. ^{14}C-Decanoic acid was incorporated equally well in all three acyl positions of TAG. The results suggest that *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia* embryos have sufficient acyltransferase activities and glycerol-3-phosphate levels to support rates of TAG synthesis in excess of those found in vivo. Consequently, the amount of TAG synthesized in these oilseeds may be in part determined by the amount of fatty acid produced in plastids.

In plants the biosynthesis of storage TAG occurs at high levels primarily in the seeds, but there is a wide range in the levels of TAG that accumulate in different plant species. For example, seeds of species of *Zea, Hordeum*, or *Pisum* usually contain less than 5% to 10% TAG by dry weight, whereas many other species such as castor accumulate over 50% TAG in seeds. The regulatory or metabolic factors that influence this very wide range of oil accumulation in seeds are currently unknown (Ohlrogge and Jaworski, 1997). Considering that over 30 reactions are required to convert acetyl-CoA to TAG, there could be many steps or genes that control the yield of end product TAG. To begin to dissect possible limiting factor(s) in the pathway of TAG biosynthesis, it is useful to conceptually divide the pathway into two parts: (a) the production of acyl chains, which occurs in plastids, and (b) the utilization of acyl chains for glycerolipid synthesis in the ER and oilbody. In the second part, the only unique enzyme for TAG synthesis is DAGAT, which is responsible for the acylation of 1,2-diacylglycerol at the *sn*-3 position (Roughan and Slack, 1982; Stymne and Stobart, 1987). Since DAGAT is located at the branch point that channels diacylglycerol to TAG synthesis, some reports have suggested that DAGAT may be one rate-limiting enzyme for the accumulation of TAG (Griffiths et al., 1988; Ichihara et al., 1988; Griffiths and Harwood, 1991; Perry and Harwood, 1993a, 1993b).

In this study we asked whether the fatty acid supply can influence the amount of TAG produced in oilseeds. If the supply of fatty acid is a limiting factor for TAG biosynthesis, then providing exogenous fatty acid to the developing embryos should increase the rate of TAG production. Unfortunately, long-chain fatty acids have very low solubility in aqueous solution, and so addition of long-chain fatty acids at concentrations sufficient to increase rates of lipid synthesis is very difficult. However, embryos of *Cuphea lanceolata, Ulmus carpinifolia*, and *Ulmus parvifolia* contain high levels of decanoic acid in their TAG (80%, 63%, and 71%, respectively), and, since decanoic acid is easily dissolved in water at millimolar concentrations, these species provided a convenient model system with which to test the influence of fatty acid supply on TAG accumulation. Such in vitro model experiments may provide a useful guide toward the selection of targets for future metabolic engineering in transgenic plants.

MATERIALS AND METHODS

Plant Materials and Chemicals

Cuphea lanceolata plants were grown in Beal garden on the Michigan State University campus (East Lansing). *C. lanceolata* plants typically begin to flower in mid-July and our experiments were performed in August. Flowers were hand-pollinated and seeds were harvested at various stages of development. After removal of the seed coat, the resulting embryos were used immediately for labeling experiments or were stored at −20°C for later lipid analysis. Embryos were also collected from two species of elm trees (*Ulmus carpinifolia* and *Ulmus parvifolia*) growing on the Michigan State University campus. Elm trees begin flowering in early May and we collected embryos from the time they were big enough to dissect until they were mature. The embryos were removed from seed coat and used for labeling experiments immediately or stored at −20°C for subsequent lipid analysis.

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Abbreviations: DAF, days after flowering; DAGAT, diacylglycerol acyltransferase; TAG, triacylglycerol.

[1-^{14}C]Octanoic acid (55 mCi/mmol), [1-^{14}C]decanoic acid (55 mCi/mmol), and [1-^{14}C]oleic acid (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis). Tritridecanoin (C13:0), L-dipentadecanoyl (C15:0) α-phosphatidylcholine, and lipase (from *Rhizopus arrhizus*) were obtained from Sigma.

Lipid Analysis

Lipids were extracted from 20 embryos at each developmental stage according to the method of Bligh and Dyer (1959). Prior to extraction, tritridecanoin (C13:0) and L-dipentadecanoyl (C15:0) α-phosphatidylcholine were added to each sample as internal standards for GC analysis. TAG was separated from polar lipids by TLC (20- × 20-cm K6 silica 60-Å plates, Whatman) in hexane:diethyl ether:acetic acid (70:30:1, v/v). TAG bands were eluted from the silica gel with chloroform:methanol (1:2, v/v). Fatty acid methyl esters from TAG were prepared by heating lipids at 90°C for 45 min in 0.3 mL of toluene and 1 mL of 10% (v/v) boron trichloride/methanol (Sigma). The resulting fatty acid methyl esters were separated and quantified by GC analysis.

Feeding Developing Embryos with Exogenous Fatty Acid

Ten pairs of *C. lanceolata* cotyledons (10 DAF) were cut in half and incubated at 28°C with gentle shaking in 200 mL of 0.1 M phosphate (pH 7.2) containing 2.1 mM [1-^{14}C]decanoic acid in the presence or the absence of 0.125 mM glycerol. The incubation buffer was changed once after 1 h of incubation. Assays were terminated by removing the incubation buffer, washing the embryos twice with water, and initiating lipid extraction. TAG was separated by TLC using a solvent system composed of hexane:diethyl ether:acetic acid (70:30:1, v/v). Labeled TAG was quantified using both an imager (Instant Imager, Packard Instruments) and liquid scintillation counting. In some experiments, other factors that may influence TAG synthesis, such as exogenous fatty acid concentration (2.1–80.1 mM), glycerol (with/without 0.125 mM), Suc (0–200 mM), and pH (6–8), were tested. Only one factor was changed in each treatment.

The position of exogenous fatty acid incorporated in TAG was determined using TAG lipase from *R. arrhizus*, which cleaves fatty acids from the *sn-1* and *sn-3* positions of TAG, and then the radioactivity remaining in the *sn-2*-monoacylglycerol was compared with free fatty acids released from the *sn-1* and *sn-3* of TAG by the action of TAG lipase. Purified TAG was dissolved in 0.5 mL of diethyl ether in 13-mL screw-cap glass tubes. One milliliter of 0.1 M Tris-HCl (pH 7.8) buffer containing 5 mM $CaCl_2$ was added to the tube, then 43,000 units of lipase was added to the bottom of the tube, bubbled in N_2, and shaken for 10 min at room temperature. Monoacylglycerol and free fatty acid products were extracted and resolved by TLC in hexane:diethyl ether:acetic acid (35:70:1.5, v/v). The radioactivity in the monoacylglycerol and free fatty acid bands on the TLC plate were quantified using an imager (Packard Instruments).

In similar experiments, 10 pairs of cotyledons from *U. carpinifolia* and *U. parvifolia* embryos at the middle stage of development were also used for feeding experiments as described above, except incubation buffer was changed every half hour.

RESULTS

Fatty Acid Deposition, Composition, and in Vivo Rate of TAG Accumulation during Embryo Development

Mature *C. lanceolata* seeds accumulate TAG, in which decanoic acid is the predominant fatty acid, reaching a level of 80 mol % (Bafor et al., 1990). Embryos were large enough to isolate at 6 DAF, and the seeds reached maturity about 20 DAF. As shown in Figure 1, TAG deposition in *C. lanceolata* embryos was linear from 8 to 12 DAF, and during this period TAG accumulated at the rate of 2.9 nmol h^{-1} $embryo^{-1}$. This result was close to the 2.3 nmol h^{-1} $embryo^{-1}$ measured by Bafor et al. (1990). During this same period, the relative amount of decanoic acid in TAG increased from 40 to 75 mol %. The fatty acid composition of

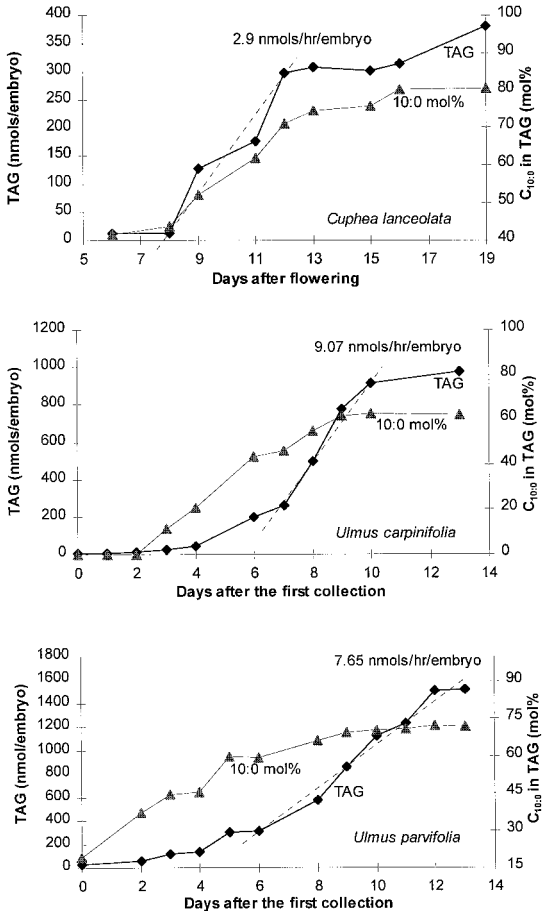

Figure 1. TAG accumulation and percentage of decanoic acid in TAG in developing embryos of *C. lanceolata, U. carpinifolia*, and *U. parvifolia*. Twenty embryos at each stage were analyzed for TAG content. The left scale represents the TAG content (♦), and the right scale represents the percent of decanoic acid in TAG (▲).

Table I. *Fatty acid composition of TAG from 20 U. carpinifolia (2,750 nmol TAG embryo^{-1}) and U. parvifolia (3,170 nmol TAG embryo^{-1}) embryos at 10 d after first collection*

Species	Fatty Acid Distribution in TAG[a]								
	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3
					mol %				
U. carpinifolia	6.2	62	4.0	3.8	6.7	0.8	7.4	6.6	1.8
U. parvifolia	7.5	71	4.2	2.6	4.2	0.9	4.9	4.4	0.6

TAG in *C. lanceolata* embryos at the middle stage of TAG accumulation (11 DAF) was similar to the results obtained by Bafor et al. (1990).

In *U. carpinifolia* and *U. parvifolia*, it is difficult to tag flowers on a daily basis because the elm trees are very tall. Therefore, embryos were collected only when they reached a stage when lipid analysis was feasible. The first collection was designated as the zero time point, and later time points were recorded as days after the first collection. The rate of TAG accumulation of *U. carpinifolia* and *U. parvifolia* embryos was linear from 7 to 10 d after first collection, and the relative amount of decanoic acid in TAG increased from 40% to 65% (Fig. 1). The fatty acid composition of TAG in both elm species at 10 d after first collection is given in Table I. At this stage the medium-chain fatty acids constituted 77% and 85% of the TAG fatty acids in *U. carpinifolia* and *U. parvifolia*, respectively.

Rates of TAG Synthesis by Developing Embryos with Addition of Exogenous Fatty Acid

Bafor et al. (1990) observed that when developing *C. lanceolata* embryos were incubated with exogenous decanoic acid and glycerol, the rate of exogenous decanoic acid incorporated into TAG was 33.9 nmol h^{-1} embryo^{-1}. Assuming that decanoic acid could be esterified to all three positions of glycerol, the rate of ^{14}C-TAG synthesis was at least 11.3 nmol h^{-1} embryo^{-1}, which is four times higher than the in vivo rate of TAG accumulation (2.9 nmol h^{-1} embryo^{-1}). To further examine and extend these results, we tested whether the addition of exogenous fatty acid alone could increase the rate of ^{14}C-TAG synthesis in *C. lanceolata* developing embryos, and whether these results could be extended to other species.

Ten pairs of cotyledons of *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*, harvested when TAG accumulation was in the linear range, were incubated in buffer containing 2.1 mM [1-^{14}C]decanoic acid with or without glycerol. As shown in Table II, in the absence of glycerol the average rates of ^{14}C-TAG synthesis were 12.5, 20.4, and 12.9 nmol h^{-1} embryo^{-1} for *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*, respectively. Compared with their respective in vivo rates of TAG accumulation, 2.9 (*C. lanceolata*), 9.1 (*U. carpinifolia*), and 7.7 (*U. parvifolia*) nmol h^{-1} embryo^{-1}, the rates of ^{14}C-TAG synthesis were approximately four times higher for *C. lanceolata* and two times higher for *U. carpinifolia* and *U. parvifolia* embryos upon addition of exogenous decanoic acid. The addition of 0.125 mM glycerol to the incubation solution did not strongly influence the rates of ^{14}C-TAG synthesis (Table II), suggesting that there is an adequate endogenous supply of glycerol for higher TAG synthesis in developing *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia* embryos. The level of ^{14}C-decanoic acid incorporated into TAG was used to calculate the rate of ^{14}C-TAG synthesis in each species (Table II). This calculation did not include the contribution derived from the endogenous de novo TAG synthesis from nonradioactive precursors, so the values given in Table II represent an underestimation of the total TAG synthesis from both exogenous and endogenous fatty acids.

The TAG derived from *C. lanceolata* embryos incubated with [1-^{14}C]decanoic acid for 1 h was treated with TAG lipase from *R. arrhizus*. The resulting *sn-2*-monoacylglycerol and nonesterified fatty acid (derived from *sn-1* and *sn-3* positions of TAG) were separated by TLC. Radioactivity was detected in both *sn-2*-monoacylglycerol and free fatty acid fractions, indicating that labeled decanoic acid was esterified to the *sn-2* as well as to the *sn-1* and *sn-3* positions of the glycerol backbone. The ratio of radioactivity from *sn-2*-monoacylglycerol to that of the free fatty acid was 1:2. This suggests that exogenous decanoic acid was equally distributed among the three positions of TAG.

We recently observed that lauric acid produced in transgenic *Brassica napus* can be subject to β-oxidation (Eccleston and Ohlrogge, 1998). If this had occurred in the embryos

Table II. *Rate of ^{14}C-TAG synthesis upon addition of 2.1 mM ^{14}C-decanoic acid*

Ten pairs of cotyledons at mid-stage of development were incubated with 2.1 mM decanoic acid in 0.2 mL of 0.1 M phosphate buffer (pH 7.2) at 28°C for 2 h in the absence (−G) or presence (+G) of 0.125 mM glycerol. The incubation buffer was changed once at 1 h for Cuphea, and every half hour for Elm. Rates of ^{14}C-TAG synthesis are calculated as nanomoles of ^{14}C-decanoic acid found in TAG (per hour) divided by three. Data represent the average of three independent experiments.

Species	Rate of TAG Synthesis
	nmol h^{-1} embryo^{-1}
C. lanceolata	
−G	12.5 ± 1.3
+G	11.1 ± 1.9
U. carpinifolia	
−G	20.4 ± 2.2
+G	21.5 ± 1.0
U. parvifolia	
−G	12.9 ± 2.0
+G	13.8 ± 1.3

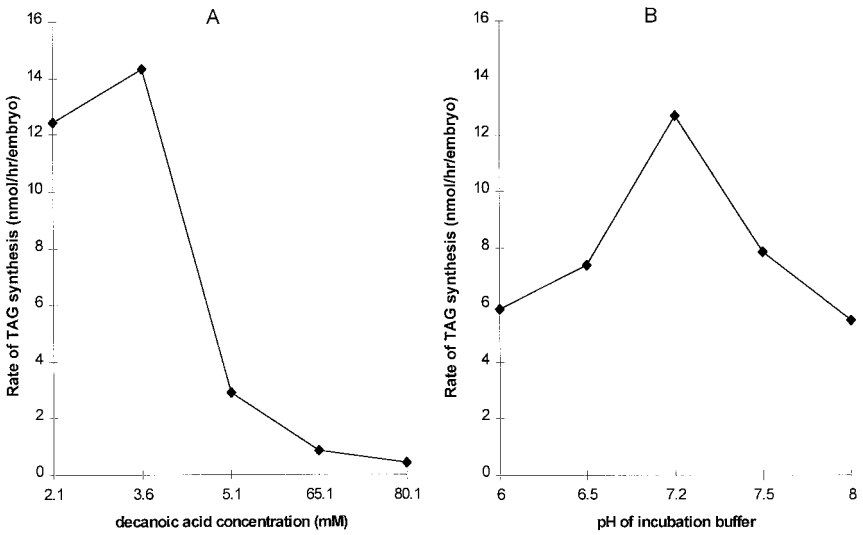

Figure 2. Rate of ^{14}C-TAG synthesis under different conditions. A, Ten pairs of cotyledons incubated in 0.2 mL of phosphate buffer (pH 7.2) with ^{14}C-decanoic acid at the concentrations indicated. B, Ten pairs of cotyledons incubated in 2.1 mM ^{14}C-decanoic acid plus 0.2 mL of phosphate buffer ranging in pH from 6 to 8.

supplemented with decanoic acid, a loss of lipid-soluble ^{14}C would have occurred and ^{14}C would have been detected not only in decanoic acid, but also in the other fatty acids isolated after the incubations. Neither event was observed and, furthermore, recoveries of added decanoic acid were at least 75% to 85%. Therefore, β-oxidation was not a major fate of the added decanoic acid.

Factors That Influence the Incorporation of Exogenous Fatty Acid into TAG

As shown in Table II, the addition of glycerol to the incubation buffer had no significant effect on the rate of ^{14}C-TAG synthesis. We also examined if other factors such as exogenous fatty acid concentrations, Suc (0–200 mM), and pH may influence the rate of ^{14}C-TAG synthesis by developing *C. lanceolata* embryos. Decanoic acid concentrations were varied over the range from 2.1 to 80 mM. As shown in Figure 2A, the highest rate of ^{14}C-TAG synthesis (14.3 nmol h^{-1} embryo^{-1}) was obtained with the addition of 3.6 mM decanoic acid. Concentrations higher than 3.6 mM decanoic acid apparently had a deleterious effect on embryos and resulted in lower rates of TAG deposition.

Developing *C. lanceolata* embryos are nonphotosynthetic tissue, so the ultimate carbon source for TAG synthesis is derived from Suc. To determine if Suc concentrations may limit TAG formation in these experiments, different concentrations of Suc (0–200 mM) were added to the basic incubation solution. Despite minor variations, the rates of ^{14}C-TAG synthesis in all samples were close to 12 nmol h^{-1} embryo^{-1}, suggesting that carbon supply in the form of Suc does not limit TAG accumulation in these short-term experiments.

Developing *C. lanceolata* embryos were also incubated under a range of pH from 6.0 to 8.0. As shown in Figure 2B, the optimal pH for TAG deposition was 7.2, whereas at pH 6.5 or 7.5, the rate of ^{14}C-TAG synthesis decreased to one-half of that at pH 7.2.

Utilization of Other Exogenous Fatty Acids by *C. lanceolata* Cotyledons for TAG Synthesis

Each of the 10 pairs of cotyledons of developing *C. lanceolata* embryos were separately supplied with 2.1 mM [1-^{14}C]octanoic acid, [1-^{14}C]decanoic acid, or [1-^{14}C] oleic acid in 200 mL of 0.1 M phosphate (pH 7.2). Triton X-100 (2.0 mM) was used to increase the solubility of the oleic acid. After 1 h of incubation, 5.8 nmol of octanoic acid, 37.5 nmol of decanoic acid, and a trace amount of oleic acid were incorporated into TAG in each embryo, respectively. One simple interpretation of this result is that one or more of the acyltransferases of *C. lanceolata* displays a strong selectivity in favor of decanoic acid (Bafor and Stymne, 1992; Vogel and Browse, 1996). However, *B. napus* embryos were also incubated with oleic acid under the same conditions and the incorporation of oleic acid into TAG was significantly lower than the in vivo rate (data not shown). Therefore, for oleic acid, low aqueous solubility or transport into the tissue may also prevent its rapid incorporation into TAG by embryos.

DISCUSSION

TAG normally accumulates to a high level only in seeds, but a metabolic understanding of the tissue specificity of oil accumulation is not yet available. One potential explanation is that DAGAT, which catalyzes the acylation of position 3 of 1,2-diacyl-*sn*-glycerol, is specifically expressed in seed. However, there are several observations that argue against this view. For example, DAGAT activity was found in spinach leaves (Martin and Wilson, 1983) and was primarily associated with chloroplast envelopes (Martin and Wilson, 1984). Roughan et al. (1987) reported that significant amounts of TAG were synthesized when palmitic acid was applied to the upper surface of expanding spinach leaves. The level of neutral lipids (mainly TAG) increased at least 3-fold during protoplast isolation from Arabidopsis leaves (Browse et al., 1988). Finally, ozone-fumigated spin-

ach leaves produced high proportions of TAG (Sakaki et al., 1990). These data together suggest that DAGAT not only occurs in leaves, but also that leaves have the ability to synthesize TAG.

Although expressed in several tissues, higher expression of DAGAT during seed development might provide one explanation for TAG accumulation in oilseeds. Ichihara et al. (1988) measured the specific activity of DAGAT from safflower in vitro and found that it was lower than expected. They concluded that the DAGAT reaction may be rate-limiting. When developing safflower and sunflower cotyledons were incubated with exogenous radiolabeled fatty acid tracers, substantial amounts of labeled fatty acids were esterified to DAG (Griffiths et al., 1988). Since DAG is the direct substrate of DAGAT, it was suggested that DAGAT could be a rate-limiting step. Perry and Harwood (1993a, 1993b) found that, when developing seeds of *B. napus* were incubated with [1-^{14}C]acetate and [2-^{3}H]glycerol, very low accumulation of the Kennedy pathway intermediates occurred apart from DAG. These results were also interpreted as indicating that DAGAT is likely to exert significant flux control over TAG accumulation.

A similar conclusion was drawn by Griffiths and Harwood (1991) from studies of TAG synthesis in cocoa. However, the accumulation of DAG might also be explained as a shortage of acyl chain supply rather than flux control at DAGAT. Because both DAG and acyl-CoA are direct substrates of DAGAT, lack of one substrate (acyl-CoA) can lead to the accumulation of the other (DAG) if the DAGAT K_m for acyl-CoA is higher than the other acyltransferases. Thus, the accumulation of DAG does not necessarily imply that DAGAT exerts flux control for TAG synthesis.

To begin to examine the limiting step(s) in TAG production for this study we considered the pathway of TAG biosynthesis in two parts. The first half can be characterized as fatty acid production inside plastids; the second half can be considered as the assembly of TAG in the ER or oilbodies (Cao and Huang, 1986; Settlage et al., 1995). If the supply of fatty acid is a limiting factor for TAG synthesis, then the addition of excess exogenous fatty acid should increase the rate of TAG synthesis. As shown in Figure 1, TAG accumulated at the rate of 2.9, 9.07, and 7.65 nmol h^{-1} embryo^{-1} in vivo for developing embryos of *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*, respectively. With addition of exogenous decanoic acid, their rate of ^{14}C-TAG synthesis was 2- to 4-fold higher than the in vivo accumulation rate. This result clearly indicates that the supply of fatty acid can be one limiting factor for TAG accumulation.

In agreement with these observations in seeds, the addition of exogenous phosphatidylcholine liposomes to *Chlamydomonas reinhardtii* cultures caused 10-fold increases in TAG accumulation (Grenier et al., 1991). Thus, it appears that the capacity of these systems for TAG accumulation is greater than actually used and that fatty acid supply, rather than the utilization enzymes may limit TAG accumulation in *C. lanceolata*, *U. carpinifolia*, *U. parvifolia*, and *C. reinhardtii*. In addition, we found that Suc and glycerol had no significant influence on the rate of ^{14}C-TAG synthesis in *C. lanceolata*. This implies that both the endogenous carbon source and the glycerol backbone are in excess and do not limit ^{14}C-TAG synthesis during these incubations. However, it is important to emphasize that such short-term incubations may not reflect factors that control overall long-term accumulation of storage oils. For example, over the time scale of seed development, many other factors such as the ability of oilbodies to accommodate increased TAG might become limiting.

In the present study exogenous decanoic acid was almost equally distributed among the three positions of TAG from *C. lanceolata*. This result implied that not only DAGAT, but also glycerol-3-P acyltransferse and lysophosphatidic acid acyltransferase could incorporate exogenous decanoic acid at rates several times above their in vivo activity with endogenous substrates. Although our studies support the concept that increased fatty acid supply can increase TAG accumulation, they do not rule out that other factors or enzyme expression levels may have a similar effect. Flux through a metabolic pathway can often be driven by either stronger source inputs and/or by stronger sinks pulling on the pathway. The observations of Zou et al. (1997) that expression of a yeast acyltransferase in *B. napus* can increase oil yields may represent an example of sink-driven increases in oil accumulation. Furthermore, we recently found that transgenic *B. napus* seeds that express very high levels of medium-chain acyl-ACP thioesterase and produce high levels of lauric acid induce the beta-oxidation pathway to degrade some of the lauric acid (Eccleston and Ohlrogge, 1998). Because oil yields are not reduced in these seeds, fatty acid synthesis apparently increased to provide a constant oil yield.

In oilseeds, fatty acids esterified to TAG can be generally divided into two groups. One (18:3, 22:1, and 18:1-HO) needs post-plastidial modification, while the other (10:0, 12:0, and 18:1) does not. Our results clearly show that the supply of fatty acids is one limiting factor for the rate of ^{14}C-TAG synthesis in *C. lanceolata*, *U. carpinifolia*, and *U. parvifolia*, which might be generalized representatives of the second category. However, for the synthesis of TAG containing high level of post-plastidially modified fatty acids, the involvement of phospholipids, desaturases, elongases, hydrolases, etc., may be additional factors that limit TAG accumulation. In addition, the observation that increased expression of acetyl-CoA carboxylase resulted in increased oil content of high-erucic rapeseed (Roesler et al., 1997) suggests that in rapeseed, in which fatty acids are modified by elongation, increased fatty acid supply can also increase TAG accumulation.

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