Diacylglycerol Acyltransferase Activity and Triacylglycerol Synthesis in Germinating Castor Seed Cotyledons

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ABSTRACT: The central importance of storage lipid breakdown in providing carbon and energy during seed germination has been demonstrated by isolating the genes encoding the enzymes involved in FA β-oxidation. In contrast, little is known about the ability of germinating seeds to synthesize TAG. We report that castor cotyledons are capable of TAG synthesis. The rate of incorporation of ricinoleic acid into TAG reached a peak at 7 d after imbibition (DAI) (1.14 nmol/h/mg) and decreased rapidly thereafter, but was sustained at 20 DAI in cotyledons and true leaves. The castor DAG acyltransferase (RcDGAT) mRNA and protein were expressed throughout seed germination at levels considerably enhanced from that in the dormant seed, thus indicating new expression. Significant degradation of the RcDGAT protein was observed after 7 DAI. The DGAT activity was found to be predominantly a function of the level of the intact RcDGAT protein, with the rate of TAG synthesis decreasing as degradation of the RcDGAT protein proceeded. A possible mechanism for the degradation of the RcDGAT protein is discussed. The induction of DGAT mRNA and protein, the capacity for TAG synthesis *in vitro* and in tissue slices, and the differing TAG composition of dormant seed TAG vs. cotyledonary TAG provide strong circumstantial evidence for active TAG synthesis by cotyledons. However, we have not yet determined the physiological significance of this capability.

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Castor plants (*Ricinus communis*) are grown for their seeds, which, at maturity, contain up to 60% of oil. Nearly 90% of the TAG FA content is ricinoleic acid, a hydroxy FA that has numerous industrial uses (1). Unlike the majority of oil crops, castor seeds store oil in a living endosperm rather than the embryo. During castor seed germination, storage oil in the endosperm is converted to sucrose *via* successive pathways of β-oxidation, the glyoxylate cycle, the partial tricarboxylic acid cycle, and gluconeogenesis for utilization by the developing embryo (2).

Although the breakdown of storage lipids is the most striking metabolic event occurring during oilseed germination (3), it is evident from data presented in previous studies that germinating seeds may also be able to synthesize TAG. In 1975, Harwood reported that all the enzymes required in FA and glycerolipid synthesis were present and active in soybean cotyledons during seed germination (4). The synthesis of TAG has also been shown to occur during seed germination of some plants, such as the pea (5), cucumber (6), and soybean (7). These observations suggest the presence of DAG acyltransferase (DGAT) in germinating seeds. Recently, we identified a cDNA encoding DGAT from castor seed (RcDGAT) based on its homology to other plant-type DGAT1 cDNA (8) and investigated the expression of the RcDGAT gene and DGAT activity in developing seeds (9). In this study we extend our examination of the RcDGAT gene and protein to cotyledons of germinating castor to elucidate the function of RcDGAT in the germination process and in plant development.

EXPERIMENTAL PROCEDURES

Growth conditions. Castor (*Ricinus communis* L.) seeds, PI 215769, were obtained from the USDA Germplasm Resources Information Network, Southern Regional Plant Introduction Station (Griffin, GA). Plants were germinated and grown in the greenhouse at temperatures ranging between 28 (day) and 18°C (night), with supplemental metal halide lighting used to provide a 15-h day length (1000–1250 μeinstein/m^2/s). The time of planting was considered to be time zero in developmental studies.

Northern blot analysis. RNA samples were extracted from germinating castor bean cotyledons and true leaves using the method of Gu *et al.* (10). Five micrograms of total RNA was applied to each lane in a 1% agarose gel with 2% formaldehyde. The probe used for hybridization was generated by PCR labeling with digoxigenin (DIG-dUTP) using RcDGAT cDNA as the template and the primers: 5′-AAGACCCCATGGCGATTCTCGAAACGCCAGAA-3′ and 5′-CTGGAGCTTCAGAACCCTCTCAA-3′. Northern analysis was performed based on the DIG Application Manual for Filter Hybridization (Roche Molecular Biochemicals, Mannheim, Germany).

Microsomal preparations. Leaf samples were collected from developing seedlings. Microsomes were isolated as described by Lu *et al.* (11). Briefly, leaves were homogenized in buffer containing 400 mM sucrose, 100 mM Hepes-NaOH (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, and a protease inhibitor cocktail tablet per 10 mL (Boehringer Mannheim, Indianapolis, IN). The homogenate was cen-

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Abbreviations: DAI, days after imbibition; DGAT, diacylglycerol acyltransferase; RcDGAT, diacylglycerol acyltransferase from *Ricinus communis*.

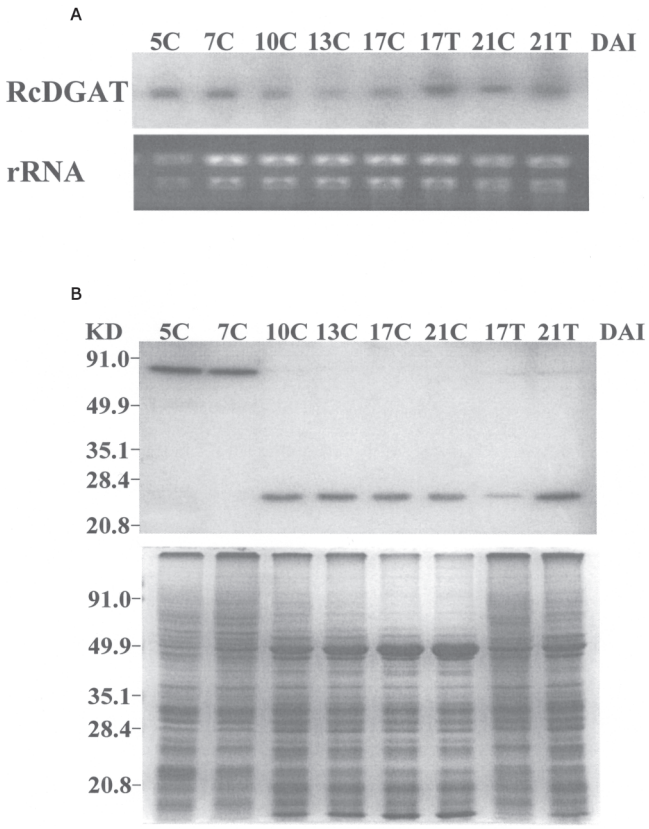

FIG. 1. Expression of castor DAG acyltransferase (RcDGAT) in germinating seedlings. RNA and protein samples were extracted from the cotyledon (C) or true leaves (T) at different stages of seed development from 5–21 d after imbibition (DAI). (A) Total RNA (5 μg) was loaded in each well of the gel for the northern blot analysis and hybridized to the RcDGAT cDNA probe. Ribosomal RNA (rRNA) was stained with ethidium bromide to show equal loading. (B) Total protein (30 μg) was used in SDS-PAGE. Top panel: The western blot was incubated with antibodies against peptide in the C-terminus of RcDGAT. Bottom panel: Duplicate protein gel stained with Coomassie Brilliant Blue R-250 is shown for equal loading of protein.

trifuged at 10,000 × *g* for 10 min to remove cell debris, and the supernatant was spun again at 100,000 × *g* for 90 min. The pellet was resuspended in the homogenizing buffer (microsomal fraction), and the protein concentration was determined using Bradford reagent (BioRad, Hercules, CA). These microsomal preparations were stored at −80°C and used for western blot and DGAT activity assay.

Western blot analysis. Thirty micrograms of microsomal protein from cotyledons and true leaves was separated by SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. The membrane was incubated with the anti-RcDGAT antibodies developed previously (9) at 1:5000 dilution, followed by horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (Amersham Pharmacia, Piscataway, NJ) at 1:3000 dilution. Horseradish peroxidase activity was visualized by chemiluminescence using the ECL kit (Amersham, Arlington Heights, IL).

In vitro DGAT assay. The DGAT assay was performed as in Cases *et al.* (12) with minor modifications. The [1-^{14}C]ricinoleoyl-CoA was synthesized according to McKeon *et al.* (13). Assay mixtures (100 μL) contained 0.1 M Tris-HCl (pH 7.0), 20% glycerol, 400 μM 1,2-diricinolein prepared previously (14), and 20 μM [^{14}C]ricinoleoyl-CoA (200,000 cpm). Reactions were started by the addition of 100 μg microsomal protein. The reactions were incubated for 15 min at 30°C with shaking and stopped by the addition of 1 μL 10% SDS. The 1,2-diricinolein was prepared as a 10 mM stock in 0.5% Tween-20. Lipids were extracted from assay mixtures using chloroform/methanol as previously described (15). The molecular species of TAG products were separated using C18 HPLC (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman Instruments Inc., Fullerton, CA) (16). DGAT activity was determined based on the ^{14}C-label incorporated into the TAG products from [^{14}C]ricinoleoyl-CoA.

Feeding cotyledons and true leaves with exogenous FA. Castor seedling leaves (0.15 g) were sliced into 1 × 5 mm^2 pieces and incubated at 25°C with gentle shaking for 2 h in 1 mL of 0.1 M sodium phosphate buffer (pH 7.2) containing FA (2 mM cold ricinoleic acid and 1.0 μCi [^{14}C]ricinoleic aicd). Assays were terminated by removing the incubation buffer and washing the samples three times with water. The lipids were extracted and analyzed as described below to measure the amount of label incorporated into TAG.

Lipid analysis. Lipids were extracted from 0.15 g of leaves at each developmental stage according to the method of Christie *et al.* (17). Samples were finally stored in methanol with 0.01% BHA at −20°C prior to HPLC analysis. Lipid classes and molecular species of TAG were separated by HPLC as we reported previously and identified by co-chromatography with a lipid standard, matching the retention times from the UV detector (absorbance at 205 nm) and flow scintillation analyzer (18).

RESULTS AND DISCUSSION

Expression of RcDGAT in leaves during castor bean germination. Northern analysis of RNA isolated from cotyledons and the first true leaves showed that RcDGAT was expressed at the transcriptional level in all samples tested (Fig. 1A). This result is in agreement with previous reports for *Arabidopsis* (11,19). In contrast, in immature castor seeds, the RcDGAT protein was not detectable until 1–2 wk after we were able to detect the RcDGAT mRNA. To determine whether the same transcription–translation "uncoupling" occurs in germinating seed, we performed western blot analysis of microsomal proteins from cotyledon and true leaf samples using antibodies against a 15-amino acid peptide in the C-terminus of RcDGAT (9). We detected a significant amount of RcDGAT protein in cotyledon samples at 5–7 d after imbibition (DAI), with most present as full-length protein (~60 kDa) as opposed to the partially degraded form prominent in developing seed (~50 kDa) (9). Previously, we found only trace amounts of mRNA and DGAT protein in the mature, dormant castor seed (9), so we conclude that their presence in the germinating cotyledon is a result of

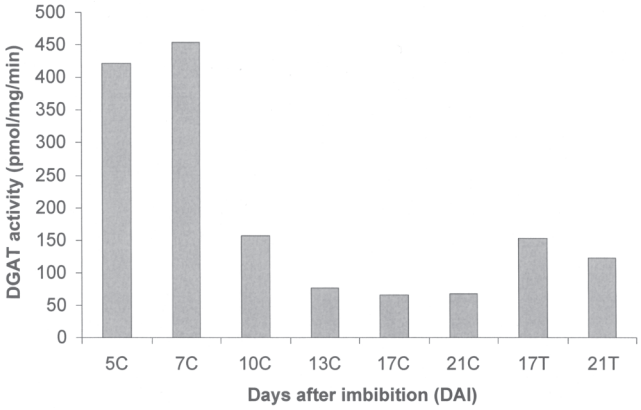

FIG. 2. DAG acyltransferase (DGAT) activities in the C and T from 5 to 21 DAI. Microsomal fractions were used for the DGAT assay. DGAT activities were measured based on the [^{14}C]-label incorporated into the TAG products. Data represent one of three independent experiments. For other abbreviations see Figure 1.

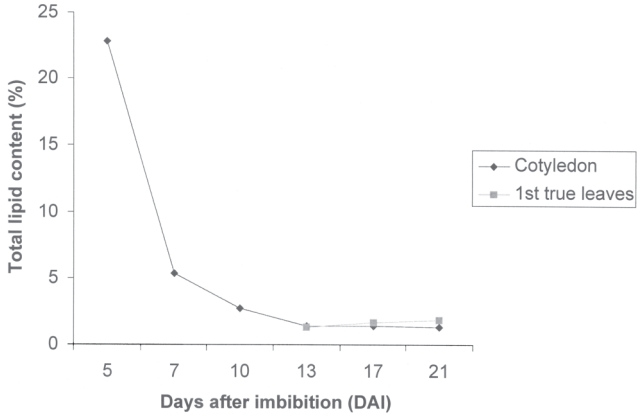

FIG. 3. Changes of lipid content in the cotyledon and true leaves during castor germination. Lipid content was measured as the percentage of total lipids to fresh weight. At each time point, 0.5 g of leaf sample was used to analyze for lipid content. For abbreviation see Figure 1.

new expression, not carryover. However, RcDGAT protein was rapidly degraded after 7 DAI. From 10 DAI, almost all RcDGAT protein in the cotyledons had degraded, whereas only small amounts remained intact in true leaf samples at 17 and 21 DAI (Fig. 1B). We do not know whether degradation of RcDGAT protein during seed germination reflects the *in vivo* situation or whether proteolysis occurred during preparation of the protein extract. However, adding protease inhibitors to the extraction buffer did not block protein degradation (data not shown).

Change of DGAT activity in germinating castor beans. To examine the effect of RcDGAT protein degradation during seed germination, we measured DGAT activity in an *in vitro* assay using microsomes isolated from cotyledons and true leaves (Fig. 2). The highest enzyme activities in cotyledons were detected at 5–7 DAI, the time at which most of the RcDGAT was still present as intact protein. Enzyme activities decreased significantly in cotyledons at 10 DAI and in true leaves at 17 and 21 DAI. In these samples, only small amounts of RcDGAT protein were intact, with most degraded to ~28 kDa. We found much lower enzyme activity in cotyledons at 13–21 DAI (about 6–7 times lower than the activity at 7 DAI), and at these times, almost all RcDGAT protein had been degraded based on western blot analysis (Fig. 1B). This indicates that DGAT activity corresponds directly to the amount of full-length RcDGAT protein. The level of full-length RcDGAT protein is likely to be regulated specifically by proteases in the seedlings since the total amount of protein produced did not differ significantly in cotyledons during seed germination (Fig. 1B).

Change of lipid content during seed germination. The germinating castor bean has a considerable supply of oil to provide energy for growth and development of the seedling. We investigated the lipid content of cotyledons during castor bean germination. The results are shown in Figure 3. We observed a dramatic decrease of the total lipid content at the early stages. The value then changed from 23% of the fresh weight at 5 DAI to 5% at 7 DAI, with a slower decline from 7–13 DAI. At 13 DAI, the lipid content was 1% and remained at this low level thereafter. The lipid contents in the first true leaves were relatively stable from their appearance at 13 (1.3%) to 21 DAI (1.8%). These results suggest that at the early stage of germination, mobilization of storage oil for transfer into the embryo dominates the metabolism in cotyledons, but beyond 13 DAI, the lipid content represents some combination of residual mobilized lipids and *de novo* synthesized lipids.

Rate of TAG synthesis in castor seedling leaves. We showed that the DGAT gene is expressed in seedling tissues. Although lipid biosynthesis is clearly important for membrane production, DGAT has generally been considered to implement a diversion of membrane lipid synthesis to oil production in seeds. Detection of DGAT activity suggests the possibility of *de novo* synthesis of TAG in cotyledons. To determine whether germinating castor bean leaves are capable of synthesizing TAG, we examined the incorporation of [^{14}C]ricinoleic acid into TAG

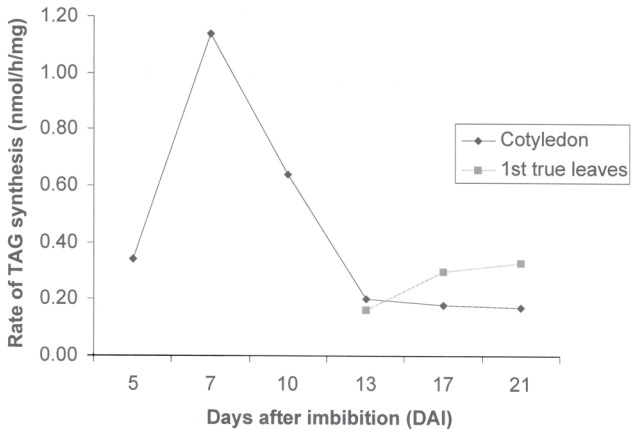

FIG. 4. Rate of ^{14}C-TAG synthesis in germinating castor seedlings. The cotyledon or true leaves (0.15 g) were sliced into small pieces and incubated with ^{14}C-labeled ricinoleic acid in 1 mL of 0.1M sodium phosphate buffer (pH 7.2) at 25°C for 2 h. The rate of ^{14}C-TAG synthesis was calculated as nanomoles of ^{14}C-ricinoleic acid found in TAG per hour in each milligram of tissue. Data represent one of three independent experiments. For abbreviation see Figure 1.

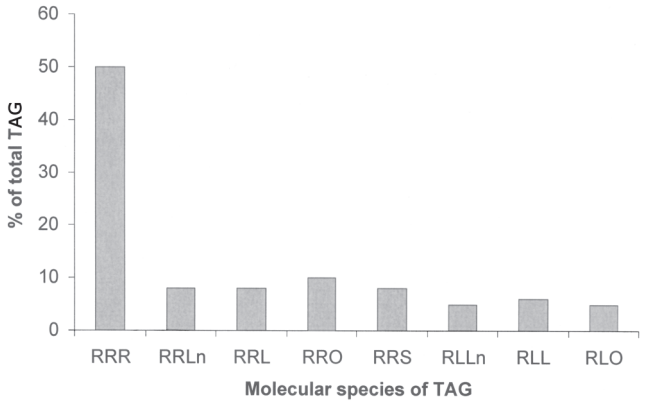

FIG. 5. Molecular species of TAG identified from castor cotyledons at 7 DAI and their contents. The data shown represent each molecular species of TAG as a percentage of total TAG. Abbreviations: R, ricinoleic acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; for other abbreviations see Figure 1.

(Fig. 4). Sliced cotyledons or true leaves were incubated in buffer containing exogenous [^{14}C]ricinoleic acid. The rate of incorporation of ^{14}C from ricinoleic acid into TAG reached a peak at 7 DAI and rapidly declined after that. At 13 DAI, the incorporation rate was one-sixth that at 7 DAI and remained at a low rate to 21 DAI. We noticed that the flux of carbon from ricinoleic acid into TAG was slightly higher in growing true leaves than in senescing cotyledons after 13 DAI. To identify the representative molecular species of TAG that incorporated ricinoleic acid, a TAG fraction was collected from cotyledons at 7 DAI and separated by HPLC on a C18 column. We observed that about 50% of TAG labeled with ^{14}C was triricinolein, indicating that germinating castor cotyledons are not only capable of TAG synthesis, but also of producing oils with characteristics similar to mature castor (Fig. 5).

Castor beans store oil in a living endosperm, which is laterally attached to the cotyledons. The stored oil is mobilized from endosperm into the cotyledons during the first several days of germination. Over the same period, the activities of many enzymes involved in the pathway, including the β-oxidation pathway, the glyoxylate cycle, the tricarboxylic acid cycle, and gluconeogenesis, increase dramatically in the endosperm and the level of sugars (mainly sucrose) increases correspondingly (3). Huang and Beevers (20) reported that the accumulation of sucrose was much higher in endosperm lacking an embryo than with the embryo because in intact seedlings, sugars were absorbed by cotyledons, then used by the growing embryo at 5 DAI. This suggests that the major product of storage oil mobilization during germinative growth is sucrose. Our observations of lipid accumulation at 5 DAI showed that cotyledons contain about 23% lipids (Fig. 3), and 30–35% of that is TAG containing ricinoleate (data not shown). At 5 DAI, the oil reserves are depleted and the endosperm detaches from the cotyledons. The origin of the ricinoleic acid and TAG in the cotyledons is not clear. Since the FA composition differs from the stored oil, TAG is probably not transported directly from endosperms. Therefore, we tested the oleoyl-12-hydroxylase activity in the microsomal fraction of castor cotyledons at 5 DAI using [^{14}C]oleoyl-CoA as substrate (21). When using the method described previously (21), we detected a low level of oleoyl-12-desaturase activity but found no hydroxylase activity, even up to 0.5 mg of microsomal protein. This result suggests that the ricinoleic acid present in the cotyledon is directly mobilized from the endosperm. The appearance of radiolabel in TAG in the feeding experiment clearly indicated that during seed germination, cotyledons have the capacity for *de novo* synthesis of TAG, with the rate of TAG synthesis reaching a peak at 7 DAI and declining rapidly. From 13 DAI, cotyledons and true leaves maintained a basal level of TAG synthesis. These results are consistent with the change of RcDGAT protein and activity during germination (Fig. 1B, Fig. 2), except the rate of TAG synthesis at 5 DAI is much lower than that predicted based on the profile of the DGAT activity assay. One possible explanation for this discrepancy is that the FA content in the cotyledon at 5 DAI is much higher than at any other time point, and labeled exogenous FA could easily be diluted, resulting in an apparently low incorporation of [^{14}C] into TAG.

We have accumulated several different lines of evidence that we believe support the idea that castor cotyledons can carry out TAG biosynthesis. The induction of DGAT mRNA and protein, the capacity for TAG synthesis *in vitro* and in tissue slices, and the differing TAG composition of dormant seed TAG vs. cotyledonary TAG provide strong circumstantial evidence for active TAG synthesis by cotyledons. However, demonstration of such activity is not proof of physiological relevance, so we can only speculate about the underlying meaning of our observations of TAG synthesis in cotyledons from germinating seeds. We suggest that at early stages of germination, when plant photoautotrophism is not fully established, the TAG synthesized and stored in leaves provides a source of energy that maintains seedling development. During the germination of castor beans, storage fats in the endosperm are converted to sucrose with high efficiency, which can result in the accumulation of sugars. In seedlings grown on high levels of sucrose, levels of FA have been observed to be much higher than seedlings grown on low levels of sucrose (22). Since energy mobilization is of importance in early seedling development, it is possible that excess sugar production is channeled into TAG for storage or utilization to prevent osmotic effects. Three enzymes, i.e., DGAT1, DGAT2 (23), and PDAT (24), have been reported to have the ability to catalyze the final step of TAG synthesis. Whether DGAT2 and PDAT are involved in the TAG synthesis we observed in extracts from germinating seeds is not known, but we believe that RcDGAT is one of the rate-limiting enzymes for TAG synthesis in castor seed (9). Still, how this enzyme is regulated during the senescence of leaves is not at all clear. Figure 1B shows unambiguously that the RcDGAT protein is expressed throughout seedling development. A significant degradation of the RcDGAT protein is observed in cotyledons and true leaves after 10 DAI, and changes in enzyme activities closely reflect those of intact protein levels. If there is TAG biosynthesis during germination, then our data

suggest that TAG biosynthesis is regulated by the level of active RcDGAT protein, and proteolytic degradation of the protein regulates its activity. This is in contrast to DGAT expressed in castor seed development, during which the DGAT is partially degraded yet remains active. RcDGAT degradation may be associated with the accumulation and release of endopeptidases from ricinosomes during seed germination. In the castor bean, cysteine endopeptidase is a marker for ricinosomes (25), organelles found exclusively in plant tissues undergoing developmentally determined programmed cell death (26). The castor bean cotyledon represents a senescing tissue, programmed to die during germination after its cellular material has been mobilized for transfer into the embryo. The decline in measurable DGAT activity suggests that targeted proteolytic degradation may be one method for controlling DGAT activity in castor tissue.

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