Relation Between Diacylglycerol Acyltransferase Activity and Oil Concentration in Soybean

Sharon B. Settlage[a], Prachuab Kwanyuen[b], and Richard F. Wilson[b,*]

[a]Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-7622, and [b]United States Department of Agriculture, Agricultural Research Service, Raleigh, North Carolina 27695-7620

ABSTRACT: Diacylglycerol acyltransferase (EC 2.3.1.20; DGAT) catalyzes synthesis of triacylglycerol from acyl-CoA and diacylglycerol. Activity of this enzyme and developmental changes in oil accumulation were estimated at various stages of seed growth in soybean germplasm with phenotypic differences in oil content. Oil deposition in seed of these genotypes followed a sigmoid pattern that was modeled to predict incremental rates of oil accumulation during seed development. A strong positive correlation was found between the estimated peak rate of oil deposition (near the mid-term of seed development) and oil concentration in mature seed. At saturating substrate levels, DGAT activity measured near the peak rate of oil deposition also was correlated positively with oil phenotype. In the latter stages of seed development, a positive correlation between estimates of enzyme activity at or below the apparent K_m for diolein and comparable oil accumulation rates was attributed to reduced synthesis of substrates and/or potential change in affinity for substrate as suggested by an increase in apparent K_m for diolein in older seed. These data indicated that DGAT activity may be a rate-limiting step in triacylglycerol synthesis. However, it is difficult to accept the idea of a single rate-limiting step at the end of a complex metabolic pathway. Because oil is a quantitatively inherited trait, several genes determine genotypic differences in oil content among soybeans. Hence, DGAT activity may be an indicator of coordinated genetic expression of gene-products in the entire glycerolipid synthetic pathway for a given genotype. In any case, results of this investigation demonstrated that genotypic differences in DGAT activity contributed to expression of genetic variation in oil content among soybean germplasm.
JAOCS 75, 775–781 (1998).

KEY WORDS: Developing seed, diacylglycerol acyltransferase, enzyme kinetics, genetic variation, *Glycine max*, modeling, oil, oil accumulation, soybean, triacylglycerol.

Although seeds of commercial soybean cultivars usually contain about 21% oil on a dry mass basis, genetic variation for this trait among accessions in the United States Department of Agriculture (USDA) Soybean Germplasm Collection ranges from 15 to 27% oil (1,2). Inheritance studies show that oil concentration in soybean is a quantitative trait determined by several genes (3). It may be assumed that these genes encode key enzymes in the glycerolipid synthetic pathway. However, it is unclear how genetic effects on particular enzyme activities in this pathway mediate the range in phenotypic variation for oil concentration that is apparent in soybean.

Soybean oil consists primarily of triacylglycerol (TG). TG is synthesized from acyl-CoA and diacylglycerol by diacylglycerol acyltransferase (DGAT), which is the final enzyme in the Kennedy pathway (4). In developing seed of various plant species, the rate of TG accumulation is correlated with acetyl-CoA carboxylase (EC 6.4.1.2; ACCase), the first enzymatic step in fatty acid biosynthesis (5–8). Although ACCase has been suggested as the rate-limiting step for fatty acid and TG synthesis (9), recent evidence shows that overexpression of genes that encode the cytosolic ACCase isozyme in rapeseed has little impact on total oil content (10).

Activities of a number of lipid-synthetic enzymes parallel oil accumulation in oilseeds. These include choline phosphotransferase, EC 2.7.8.16 (11), acyl-ACP thioesterase, EC 3.1.2.14 (12), phosphatidate phosphatase, EC 3.1.3.4 (13), and DGAT (14,15). Expression of any or all of these enzymes may have a pivotal role in regulating rates of oil accumulation and determining genotypic differences in seed oil content. This investigation was conducted to determine whether variation among soybean accessions that differ in oil content was associated with genotypic differences in DGAT activity and to show how this enzyme activity related to developmental changes in oil deposition.

MATERIALS AND METHODS

Plant material. Six soybean [*Glycine max* (L.) Merr.] genotypes in the same maturity group that varied in seed oil concentration were used in this investigation. These lines included two low-oil genotypes, NC-104 and NC-112 (16); PI407994, a plant introduction with normal oil concentration; and three high-oil lines, N88-480 (17), PI371611, and PI324924. Plant introductions were obtained from Dr. R.L. Nelson, curator

*To whom correspondence should be addressed at 4114 Williams Hall, 100 Derieux St., North Carolina State University, Raleigh NC 27695-7620.
E-mail: rwilson@cropserv1.cropsci.ncsu.edu

of the USDA Soybean Germplasm Collection (Urbana, IL). Plants were grown at the Central Crops Research Station (Clayton, NC). The developmental stage of harvested seed was determined in days after flowering (DAF) by comparison with pods and seed of known age in each genotype.

Tissue analyses. Mass and oil content were determined in seed (20 to 30 g fresh weight) harvested at various stages of development. Oil concentration was measured by wide-line nuclear magnetic resonance (18). Oil content was expressed as TG on a molar basis by assuming a molecular mass of 872 daltons. Oil deposited in seed on a daily basis was predicted from Equation 1 for a sigmoid growth curve (19),

$$\text{seed oil}_{(t)} = \left[G_{\max}\, e^{k(G_{\max})(t - T_{1/2})}\right] \Big/ \left[1 + e^{k(G_{\max})(t - T_{1/2})}\right] \qquad [1]$$

where seed $\text{oil}_{(t)}$ is oil content (mmol TG · seed^{-1}) at developmental time t; $G_{\max}$ is oil content at seed maturity; k is an empirically derived constant; $T_{1/2}$ is the estimated DAF when half of the final seed oil content is achieved and also an estimate of the date when the maximal rate of oil accumulation occurred. Parameters were optimized to each data set with an iterative fitting procedure developed in Microsoft Excel v3.0. Incremental rates, $\text{rate}_{(t)}$, of oil accumulation (mmol TG · $\text{seed}^{-1}\text{day}^{-1}$) were determined from the derivative of Equation 1, which is given by Equation 2, where

$$\text{rate}_{(t)} = \left\{\left[\left(G_{\max}^{2}\right) k e^{k(G_{\max})(t - T_{1/2})}\right] \Big/ \left[1 + e^{k(G_{\max})(t - T_{1/2})}\right] \cdot \left[1 - e^{k(G_{\max})(t - T_{1/2})}\right] \Big/ \left[1 + e^{k(G_{\max})(t - T_{1/2})}\right]\right\} \qquad [2]$$

The peak rate of oil accumulation, $\text{rate}_{(T_{1/2})}$, may be determined by substituting $T_{1/2}$ for t. Then Equation 2 simplifies to Equation 3:

$$\text{rate}_{(T_{1/2})} = \left[\left(G_{\max}^{2}\right)k\right]/4 \qquad [3]$$

Duration in days of the linear phase of oil accumulation was determined from the interval between dates in the normal distribution from Equation 2 that were equivalent to 75% of the peak rate. These dates were calculated by setting Equation 2 equal to 75% $\text{rate}_{(T_{1/2})}$ and solving for t. This approach was in agreement with other methods to define the linear phase of seed growth (20). In its quadratic form,

$$-3e^{2[k(G_{\max})(t - T_{1/2})]} + 10e^{[k(G_{\max})(t - T_{1/2})]} - 3 = 0 \qquad [4]$$

Equation 3 may be solved for t at 75% $\text{rate}_{(T_{1/2})} = T_{1/2} \pm [1.09/(G_{\max}\, k)]$. Duration then is the difference in days between the two dates.

In-vivo *acetate saturation kinetics.* Whole cotyledons (0.5 g fresh weight) were incubated at 25°C in 3 mL 0.2 N MES buffer, pH 5.5, with 5 μCi [2–^{14}C]acetate (57 mCi · mmol^{-1}) plus one of three levels (0.0, 1.0, or 10.0 mol) of potassium acetate. Reactions were terminated at 2 h. Glycerolipid extraction and analysis followed the methods described by Wilson and Kwanyuen (21). Radioactivity in glycerolipid fractions was expressed relative to the specific activity of total acetate added to each reaction. The resultant data emulated sigmoidal curves from which kinetics of the synthetic reactions at saturating substrate levels were interpreted with Hofstee plots (22). Development and original application of this procedure were documented by Moorman (23).

Extraction of DGAT from developing seed. Seeds were excised from pods, which had been kept on ice, within 1 h of harvest. Homogenates were prepared from a known number of seeds (*ca.* 15 to 20 g fresh tissue) with addition of 0.6 M sucrose to the homogenizing buffer (24). After filtration through eight layers of cheesecloth, the homogenate was centrifuged for 20 min at 16,000 × *g*. The supernatant was applied to a DEAE Bio-gel A (Bio-Rad, Richmond, CA) column, which was equilibrated with 25 mM Bicine-NaOH (pH 8.0), 10 mM disodium EDTA, and 0.4 M sucrose. With the same buffer, DGAT activity eluted in the void volume and exhibited a 2- to 5-fold enrichment in specific activity. About 90% of total DGAT activity was recovered from the homogenate. All procedures were performed at 4°C.

DGAT assay. DGAT activity was estimated with partially purified enzyme by incorporation of [^{14}C]oleoyl-CoA (60 mCi · mmol^{-1}) into TG as described previously (24) except that $MgCl_2$ was omitted from the assay buffer. Protein was estimated by the Bradford method (25) with Bio-Rad protein dye and bovine gamma globulin as the standard. All data were reported as means of three assays with a given extract.

RESULTS

Accumulation of oil during seed development. The six soybean genotypes selected for this study ranged from 17.0 to 23.9% oil (dry mass basis) at maturity (Table 1). Variation occurred among these genotypes in mass of mature seed (127 to 210 mg seed^{-1}). Although a positive correlation ($r^2 = 0.84$) between seed mass and oil concentration appeared among these genotypes, there is no significant association between these traits among all accessions in the USDA Soybean Germplasm Collection (3). Thus, this observation was considered to be coincidental and unrelated to the biochemical and genetic effects that determine oil content.

Oil content (μmol TG · seed^{-1}) at various stages during seed development was determined for each genotype. These data were used to estimate cumulative daily oil content from Equation 1 (Materials and Methods section). The application of this approach has been reported for other plant species (26), and accuracy of prediction ($r^2 = 0.96$) was confirmed with an extensive data set taken from the literature (27). Calculated and actual oil accumulation or deposition (mmol TG · seed^{-1}) during development of genotypes in this investigation is shown in Figure 1.

The derivative of the fitted equation was used to estimate incremental rates of oil accumulation over the reproductive growth period. These data presented a normal distribution where the peak oil accumulation rate (μmol TG · $\text{seed}^{-1}\text{day}^{-1}$) occurred at the midpoint of the linear phase of concurrent oil deposition. As shown in Figure 2, rate curves for the respec-

TABLE 1
Parameters That Define the Oil Phenotype of Selected Soybean Germplasm

	Mature seed			Oil accumulation characteristics[a]		
	Dry mass	Oil		$T_{1/2}$	Peak rate	Duration
Genotype	(mg · seed^{-1})	Dry mass (%)	μmol TG · seed^{-1}	(DAF)	(μmol TG · seed^{-1}d^{-1})	(d)
NC-104	148.0	17.4	29.6	36.4	1.6	10.3
NC-112	126.9	17.0	24.7	38.6	0.9	15.8
PI407994	183.3	20.1	42.3	43.1	2.3	10.0
PI371611	186.8	22.9	49.1	36.5	2.8	9.6
PI324924	210.4	23.9	57.7	37.7	3.1	10.0
N88-480	182.0	23.1	48.2	44.4	2.0	13.6
LSD0.05	22.1	2.2	9.2	2.5	0.6	1.9

[a]Oil accumulation parameters were estimated from the fitted models shown in Figure 2; $T_{1/2}$, midpoint of oil accumulation; Peak rate, rate at $T_{1/2}$; Duration, length of the linear phase of oil accumulation. DAF, days after flowering.

tive genotypes varied in amplitude and width. Because the total area under each curve represented the amount of oil in mature seed, the peak rate of oil accumulation may be estimated by solving the derivative for $T_{1/2}$, the period in DAF when 50% of total oil in mature seed was achieved. Duration of the linear phase of oil synthesis was estimated from the days between points on these distributions when incremental rates were at least 75% of the peak rate. Hence, peak rate · duration equaled the amount of oil produced during the linear phase of oil synthesis.

A strong positive correlation (r^2 = 0.85) was found between maximal or peak rates of oil accumulation and oil content of mature seed (Table 1). However, the correlation may be weaker in genotypes with a relatively longer linear growth phase. This was apparent in the high-oil genotype N88-480 and the low-oil genotype NC-112, where longer duration of the linear phase was associated with a lower maximal rate of oil accumulation, compared to lines with similar phenotype. Although the biological basis for this phenomenon is un-

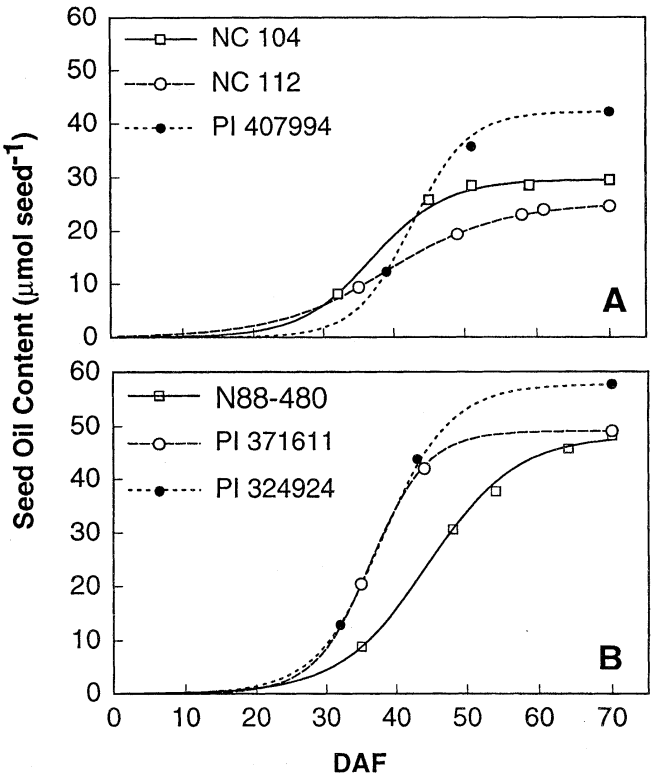

FIG. 1. Genotypic differences in oil accumulation during soybean seed development. Concurrent oil accumulation was estimated from measured data by using Equation 1 (see Materials and Methods section). (A) Genotypes with low or normal oil content; (B) genotypes exhibiting high oil content. Measured oil contents are plotted on the curve generated for each genotype. DAF, days after flowering.

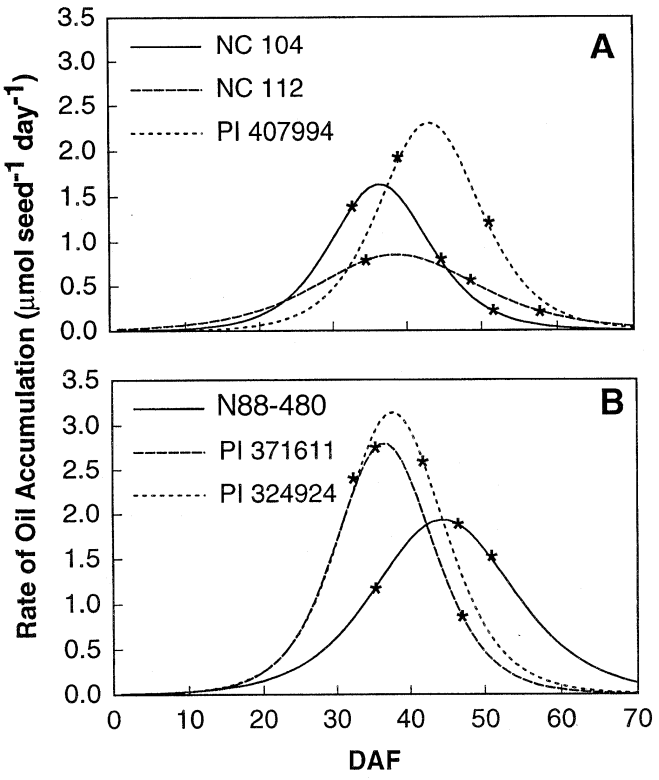

FIG. 2. Genotypic differences in rate of oil accumulation during soybean seed development. Incremental oil accumulation rates were estimated from data in Figure 1 by using Equation 2 (see Materials and Methods section). (A) Genotypes with low or normal oil content; (B) genotypes exhibiting high-oil content. Asterisks denote tissue harvest dates. For abbreviation see Figure 1.

TABLE 2
Kinetics of Acetate Metabolism in Soybean Cotyledons at 35 DAF

	PI 324924		N88-480		NC-104	
Acetate	TG[a]	TL	TG	TL	TG	TL
(mM)	(nmol Acetate · h^{-1} · $gDWT^{-1}$)					
0.03	8.8	59.3	6.2	43.6	7.0	41.4
0.36	88.9	607.4	63.4	441.6	66.9	393.9
3.36	301.1	2033.2	229.5	1623.5	192.9	1131.8
V_{max}[b]	422.7	2862.5	322.9	2367.4	251.1	1468.9
K_m	1.4	1.4	1.5	1.6	1.0	1.0
R^2	0.999	0.999	0.999	0.999	0.999	0.999

[a]TG, triacylglycerol; TL, total lipid; gDWT, gram dry weight. For other abbreviation see Table 1.
[b]Kinetics from Hofstee plots.

known, the apparent inverse association between these parameters contributed to genotypic variation in oil content.

Glycerolipid synthesis from acetate. Glycerolipid synthetic capacity of soybean cotyledons at 35 DAF was estimated from *in-vivo* acetate saturation kinetics for PI 324924 and N88-480 (high-oil genotypes) and NC-104 (low-oil genotype). These experiments provided estimates of the kinetics of glycerolipid synthesis, unlimited by substrate availability, at a date near the peak rate of oil accumulation (Table 2). A strong correlation (r^2 = 0.99) was found between the apparent V_{max} for TG synthesis from acetate and the predicted peak rate of TG accumulation among these genotypes. A strong correlation (r^2 = 0.99) also was found between the apparent V_{max} for total glycerolipid synthesis from acetate and oil content of mature seed. These data established a direct relation between glycerolipid deposition and synthesis that revealed a metabolic basis for genotypic differences in oil content. These associations implied that kinetics of certain enzymes in the glycerolipid synthetic pathway differed among and within high- or low-oil soybeans. Although the activity of several lipid-synthetic enzymes has been shown to parallel oil accumulation in various oilseeds, evidence is needed to show that differences in enzyme kinetics mediate genetic variation in oil content. This investigation considered the role of DGAT in determining the oil phenotype of soybean germplasm.

DGAT activity during seed development. DGAT activity at various stages of seed development in high- or low-oil genotypes was measured in separate reactions that contained 0.5 to 8 μM [^{14}C]oleoyl-CoA with 1 mM diolein or 0.05 to 1 mM diolein with 8 μM [^{14}C]oleoyl-CoA. Because DGAT catalyzes a bireactant reaction, the kinetics for both substrates were considered important to this analysis. Apparent V_{max} and K_m for oleoyl-CoA at saturating levels of diolein, and for diolein at saturating levels of oleoyl-CoA, were derived from Hofstee plots (22). As demonstrated in Figure 3, kinetics with each substrate gave similar V_{max} for DGAT from a given genotype at comparable stages of seed development. Results for all genotypes are reported in Table 3.

Regression analyses among data in Table 3 were performed

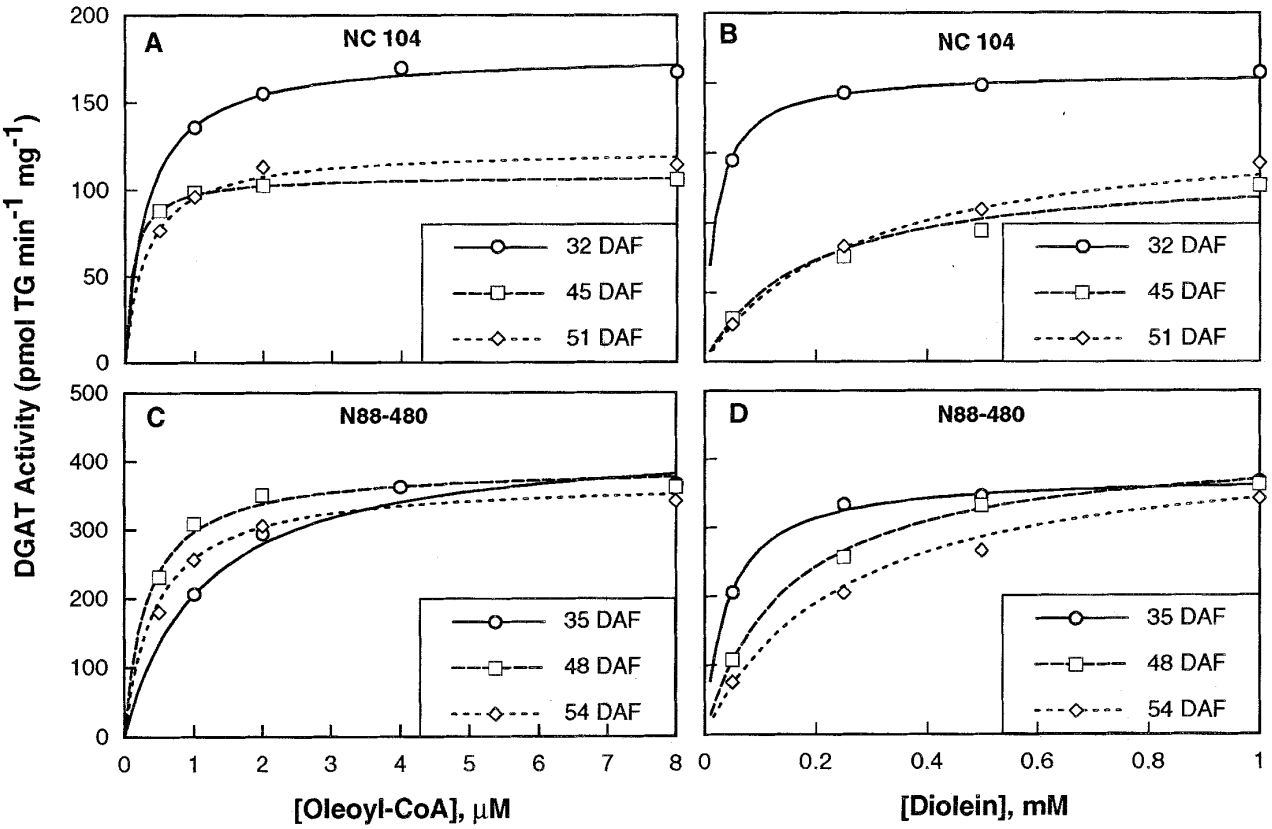

FIG. 3. Genotypic differences in diacylglycerol acyltransferase (DGAT) kinetic activity during soybean seed development. Apparent V_{max} and K_m for oleoyl-CoA and diolein were determined from Hofstee plots. Kinetics are shown for NC-104, a low-oil genotype, and N88-480, a high-oil genotype. For other abbreviation see Figure 1.

TABLE 3
DGAT Kinetics During Seed Development in Selected Soybean Germplasm

Genotype	Seed age (DAF)	TG synthesis V_{max} (pmol · mg^{-1}min^{-1})	TG synthesis V_{max} (nmol · seed^{-1}min^{-1})	K_m(18:1–CoA) (μM)	K_m (diolein) (mM)	DGAT activity at 20 μM diolein (nmol TG · seed^{-1}min^{-1})	Rate of oil accumulation[b] (μmol TG · seed^{-1}day^{-1})
NC-104	32[a]	172	0.4	0.30	0.02	0.19	1.30
	45	110	1.2	0.11	0.18	0.12	0.74
	51	130	1.4	0.29	0.27	0.10	0.24
NC-112	35[a]	156	0.3	0.76	0.02	0.14	0.80
	49	48	0.4	0.40	0.06	0.09	0.53
	58	80	0.7	0.50	0.21	0.06	0.21
PI407994	39[a]	538	1.4	0.48	0.07	0.32	1.90
	51	274	2.2	0.44	0.14	0.27	1.18
PI371611	35[a]	698	1.9	0.72	0.08	0.38	2.72
	48	540	3.1	0.80	0.27	0.22	1.45
PI324924	32	256	0.7	0.57	0.03	0.28	2.19
	43[a]	340	2.7	0.86	0.12	0.35	2.29
N88-480	35	403	0.7	1.04	0.04	0.20	1.17
	48[a]	409	2.7	0.32	0.15	0.33	1.79
	54	384	2.8	0.44	0.25	0.24	1.10
$LSD_{0.05}$		68	0.4	0.09	0.03	0.04	0.27

[a]Harvest date nearest the midpoint of oil accumulation.
[b]Estimated rate of oil accumulation at the date of harvest. DGAT, diacylglycerol acyltransferase; $LSD_{0.05}$, least significant difference at a level of $P = 0.05$. For other abbreviations see Tables 1 and 2.

to determine the strength of the relation between DGAT activity and the rate of oil deposition as seed developed, and to establish a relation with final seed oil content. Specific activities (pmol TG · mg reaction protein^{-1}min^{-1}) generally were greater prior to or near the peak rate of oil accumulation and declined thereafter. When expressed on a seed basis, V_{max} for DGAT increased as seed matured. There was a positive correlation between these data and concurrent oil deposition within and among genotypes ($r^2 = 0.76$). Although that association may be a function of genotypic differences in seed mass, DGAT activity (nmol TG · seed^{-1}min^{-1}) measured nearest the date of peak oil accumulation (shown in Table 2) was positively correlated with both oil concentration ($r^2 = 0.95$) and content ($r^2 = 0.90$) of mature seed (Figure 4). This finding demonstrated that genotypic differences in DGAT activity were associated with expression of high- and low-oil traits in soybean.

It was curious that DGAT activity (nmol TG · seed^{-1}min^{-1}) for all genotypes increased during the period when oil accumulation rates were in decline. However, these estimates of V_{max} assumed both substrates were at saturating levels. Given reports of reduced activity of enzymes involved in fatty acid, acyl-CoA, and diacylglycerol synthesis during that period (11–14), it is unlikely that *in-vivo* substrate levels would be sufficient to sustain maximum DGAT velocities. Indeed, *in-vivo* acetate saturation kinetics showed reduced glycerolipid synthetic capacity in soybean cotyledons at 45 DAF, after the peak rate of TG accumulation (23). This suggested that all enzyme activities associated with the glycerolipid synthetic pathway declined during the latter stages of soybean seed development. To expand on that assumption, DGAT velocity (nmol TG · seed^{-1}min^{-1}) at 20 μM diolein was calculated from the derived K_m in each treatment (Table 3). As shown, DGAT activity at a low concentration of diolein decreased during seed development and had a strong positive correlation with rates of oil accumulation predicted at

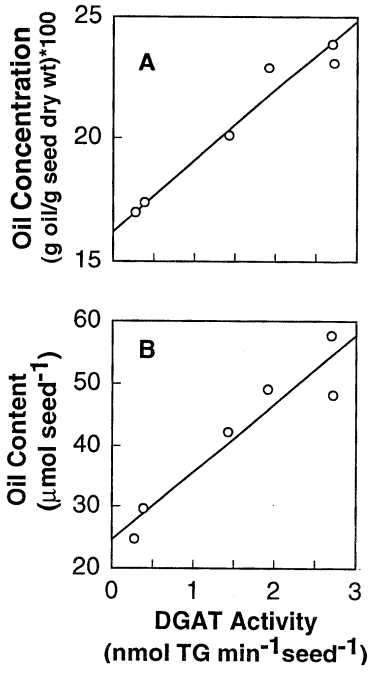

FIG. 4. Relation of DGAT activity with oil phenotype in soybean. V_{max} for DGAT, measured at or near the date of the peak oil accumulation rate in high- and low-oil genotypes, were regressed against (A) oil concentration in mature seed ($r^2 = 0.95$) or (B) oil content of mature seed ($r^2 = 0.90$).

each sampling date ($r^2 = 0.89$). Taking into account the probable availability of diolein or possible change in affinity for diolein during the latter stages of seed development, DGAT activity paralleled incremental oil accumulation rates throughout reproductive growth.

DISCUSSION

Oil deposition during seed development was sigmoidal for each of the soybean germplasm studied. Hence, oil accumulation could be modeled mathematically to assess genotypic differences in the incremental rates and duration of the linear phase of oil accumulation during seed growth. The nonlinear equations used in this approach were preferred above fitting linear equations for seed growth analysis (28–30) because they enabled estimates of time-specific rates during seed development. The nonlinear model revealed that oil concentration and content primarily were a function of the peak rate of oil accumulation. However, there were examples in both high- or low-oil genotypes where either a lower rate in conjunction with longer duration of the linear growth phase or vice versa achieved the same oil phenotype. This information significantly broadened current concepts of biological regulation of oil content in soybean.

In general, an enzyme activity in the lipid-synthetic pathway that is highly correlated with the rate of oil synthesis may be regarded as a potential rate-limiting step. Results of this investigation suggest such a role for DGAT. Regression analyses indicate that up to 95% of the variation in oil concentration among the genotypes studied was attributed to genotypic differences in the apparent V_{max} for DGAT measured during the first half of the linear phase of oil deposition, and DGAT velocities calculated at a limiting diolein concentration paralleled the decline of oil accumulation in the latter half of that phase. However, it is difficult to accept the idea of a single rate-limiting step at the end of a complex metabolic pathway, especially in view of saturation kinetics for total glycerolipid synthesis from acetate. Because oil is a quantitatively inherited trait, it is highly probable that DGAT activity is only one of several factors that determine genotypic differences in oil content among soybeans. Hence, DGAT activity may be an indicator of coordinated genetic expression of gene-products in the entire glycerolipid synthetic pathway for a given genotype. In any case, results of this investigation demonstrated that DGAT activity was associated with the expression of genetic variation of oil content in soybeans.

ACKNOWLEDGMENTS

This research represents a cooperative investigation between the United States Department of Agriculture, Agricultural Research Service and the North Carolina Agricultural Research Service at Raleigh, NC. Funding was received in part from the United Soybean Board, project number 5045.

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[Received June 24, 1997; accepted January 21, 1998]