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Fatty Acid Specificity and Selectivity of the Chloroplast *sn*-Glycerol 3-Phosphate Acyltransferase of the Chilling Sensitive Plant, *Amaranthus lividus*

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ABSTRACT

Chilling sensitivity of plants is strongly correlated with the presence of high levels of a species of chloroplast phosphatidylglycerol that contains two saturated fatty acids. The most straightforward synthetic pathway for this lipid would require the primary acylation of *sn*-glycerol 3-phosphate (G3P) with a saturated fatty acid (palmitic acid) rather than with oleic acid, an unsaturated acid. This selective incorporation would differ markedly from the reported properties of the chloroplast G3P acyltransferases of pea and spinach, two chilling resistant plants and thus we have studied the chloroplast G3P acyltransferase of *Amaranthus lividus*, a chilling sensitive plant. In contrast to our results and those of others (M. Frentzen *et al.* 1983 Eur J Biochem 129: 629–636 and previous work) with the pea and spinach enzymes, the amaranthus chloroplast G3P acyltranferase did not select oleic acid donors from a mixture of oleic and palmitic acid donors (either coenzyme A or acyl carrier protein thioesters). Instead the fatty acid composition of the synthesized 1-acyl G3P faithfully reflected the composition of the acyl donor mixture. However, the amaranthus enzyme did strongly select against incorporation of stearic acid. The properties of the amaranthus G3P acyltransferase are consistent with this enzyme having the major role in synthesis of the disaturated phosphatidylglycerol species.

Chilling sensitivity of a large number of taxonomically diverse plants has been correlated with the presence of high levels of a phosphatidylglycerol species containing two saturated fatty acids (disaturated PG[2]). In contrast, plants resistant to chilling contain only low levels of disaturated PG (15, 16, 22). Disaturated PG undergoes a thermotropic phase separation at 30 to 40°C which is thought to disrupt normal function of the chloroplast membrane (17). The unsaturated PG species found in the chloroplasts of chilling resistant plants begin phase separation at much lower temperatures (<15–20°C) (17). Only PG of the major leaf glycerolipid classes undergoes a significant phase separation (17).

Roughan (24) has recently shown that purified chloroplasts of the chilling sensitive plant, *Amaranthus lividus*, synthesize abundant amounts of disaturated PG *in vitro*. The most straightforward mechanism that accounts for the fatty acid compositions of the PG, PA, and LPA molecules synthesized from [^{14}C]acetate by these chloroplasts is that the disaturated PG is formed by acylation of G3P with a saturated fatty acid followed by a second acylation of the resulting LPA to form PA which in turn is converted to PG. It therefore seemed that the amaranthus enzyme catalyzing the first acylation, G3P acyltransferase, must have a markedly different specificity than the enzymes purified from pea and spinach, two chilling resistant plants. Bertrams and Heinz (3) and Frentzen *et al.* (8) have shown that the pea and spinach enzymes specifically acylate position one of G3P with oleic acid, thus leading to the unsaturated PG species found in chloroplasts of these plants. These enzymes discriminate very strongly against the incorporation of palmitic acid into position one of G3P (3, 8).

In this paper we report the characterization of the G3P acyltransferase activity of amaranthus chloroplasts and compare the substrate specificity and selectivity of this enzyme with those of pea and spinach chloroplasts.

MATERIALS AND METHODS

Materials. Nonradioactive acyl-CoA esters were synthesized by the acyl-imadazole method (13) modified by using an imidazole buffer (7). Radioactive acyl-CoA esters were synthesized using acyl-CoA synthetase (Sigma) as described by Bertrams and Heinz (3) except that Triton X-100 was used rather than Tween (which contains free fatty acids). All acyl-CoA esters were purified as described by Bishop and Hajra (4) and were pure as determined by their UV spectra and adenine to thioester ratios. ACP was purified from *Escherichia coli* K-12 (20). Acyl-ACP substrates were synthesized using acyl-ACP synthetase and purified by hydrophobic interaction chromatography (21). ^{32}P-labeled G3P was synthesized as described by Chang and Kennedy (5) except that carrier-free ^{32}Pi (100–250 μCi) and 3 μmol of ATP were used and the G3P was purified by elution from Dowex AG-1-X4 (formate form) with 1.5 M HCOOH (2). The yield of G3^{32}P varied from 55 to 90% based on ^{32}Pi. [2-^{3}H]G3P was synthesized using nonradioactive ATP. PA and LPA were obtained by phospholipase digestion of egg phosphatidylcholine (9). 2-Monoacyl glycerol was synthesized from olive oil using *Rhizopus* lipase and a portion was allowed to isomerize to 1-monoacyl glycerol.

Enzyme Assays. Acylation of G3P was assayed either by the filter disk assay of Goldfine (10) (modified by using 1% TCA in the last wash) or by extraction into chloroform following acidification of the assay mixture (3). Essentially identical values were given by the two methods except that the filter disc assay gave 2- to 5-fold lower background values. The assay mixtures (40 μl) consisted of a 50 mM (pH 7.6) buffer (usually K-phosphate but sometimes potassium Hepes or potassium Tes) 0.11 mM ^{32}P-

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[2] Abbreviations: PG, phosphatidylglycerol; G3P, *sn*-glycerol 3-phosphate; PA, phosphatidic acid; LPA, lysophosphatidic acid (monoacylglycerol 3-phosphate); ACP, acyl carrier protein.

labeled G3P ($1-10 \times 10^8$ dpm/μmol) acyl donors (usually 90 μM acyl-CoA or 5–25 μM acyl-ACP) and enzyme. The acyl-CoA mixtures also contained 0.25 mg/ml BSA (fatty acid-free, from Miles Laboratories). Following incubation (15–40 min at 22–25°C) the reaction mixtures were either extracted with chloroform-methanol or pipetted onto a filter disc (previously soaked in 0.1 M *rac*-G3P in 20% TCA and dried). After 3 to 5 washes (10) the disks were counted in 0.1 N HCl by Cerenkov radiation. One unit of acyltransferase activity was 1 pmol of G3P incorporated per min. Protein was determined by a microbiuret procedure standardized with BSA (28). The substrate concentration chosen gave maximal activities with the amaranthus G3P acyltransferase. All assays were done under conditions giving a linear response with respect to protein concentration and time.

Thioesterases active on acyl-CoA or acyl-ACP esters were assayed using ^{14}C fatty acid labeled compounds as described previously (20, 28). Phosphatase activity was assayed by precipitation of ^{32}Pi as the phosphomolybdate complex (29).

Enzyme Preparations. Chloroplasts were prepared from expanding leaves of hydroponically grown plants. The method of Cerovic and Plesnicar (6) was used for pea (*Pisum salivum*) and spinach (*Spinacia oleracea*) and the methods of Jenkins and Russ (11) for amaranthus. These preparations typically were capable of rates of O_2 evolution of 150 to 200 μmol h^{-1}/mg^{-1} Chl. O_2 evolution was dependent on HCO_3^- in spinach chloroplasts, on 50 μM 3-phosphoglyceric acid and 50 μM oxalacetic acid in pea and on 0.2 mM 3-phosphoglyceric acid in amaranthus. Purified amaranthus chloroplasts were washed once in 25 mM potassium Hepes buffer (pH 7.8) containing 0.33 M sorbitol and 5 mM EDTA. Chloroplast pellets were resuspended in 5 to 10 volumes of 10 mM K-phosphate (pH 7.6) for lysis by osmotic shock (1). However, since amaranthus chloroplasts were incompletely lysed by this procedure, these preparations were then slowly frozen and thawed (1) in the hypotonic buffer to complete lysis. Stromal fractions were obtained by low speed centrifugation for 2 min at room temperature in an Eppendorf microfuge (about 12,000*g*) followed by ultracentrifugation at 150,000 to 200,000*g* (average for 2 h at 4°C. Ultracentrifugation was done in a 0.6 ml Eppendorf centrifuge tube placed in a Beckman SW41 rotor bucket half filled with cold water. The supernatants were recovered using a micropipettor. Ultracentrifugation was necessary to decrease the 1-monoacyl G3P acyltransferase activity of the pea and spinach extracts so that 70 to 90% of the product was LPA (rather than PA). However, ultracentrifugation usually gave only a small increase in LPA accumulation in amaranthus extracts over that of the low speed supernatant.

Lipid Analyses. After counting the Cerenkov counting solution was removed by aspiration, the filters were dried, and the phospholipids eluted with 2 ml of $CHCl_3$:CH_3OH (1:1, v/v) followed by 2 ml of CH_2Cl_2. Carrier phosphatidic acid (5–10 μmol) was added to the pooled eluates (recovery >90%) and the solvent evaporated under N_2. The dimethyl derivatives were obtained by treatment with CH_2N_2 (19) in 10% CH_3OH in diethylether and were resolved into saturated and unsaturated species by TLC. The plates were 250 μm 10% (w/w) $AgNO_3$-silica gel G plates (stored over a saturated solution of $CaCl_2$) developed in $CHCl_3$:CH_3OH:acetone (94:5:3; v/v/v).

The dimethyl derivatives were also analyzed by reverse phase TLC on silica gel G plates dipped in 12.5% paraffin in petroleum ether. After evaporation of the solvent and sample application the plates were developed in acetonitrile:acetone:water (2:1:1, by volume). In some cases the unmodified acyl G3P compounds were resolved into lysophosphatidic acid (R_F 0.5) and PA (R_F 0.93) by TLC on plates of silica gel G containing 5% (w/w) sodium bisulfite developed in acetone:benzene (3:2, v/v) (23).

It should be noted that the phospholipids isolated from control incubations containing only a single acyl donor (palmitoyl, oleoyl, or stearoyl) gave three chromatographic spots rather than the two (LPA and PA) spots expected. The two spots of greatest mobility corresponded to dimethyl PA and dimethyl LPA. The third spot migrated just behind the dimethyl LPA spot on Silica Gel G and $AgNO_3$-Silica Gel G TLC and thus seems slightly more polar than dimethyl LPA. We have tentatively identified this spot as the monomethyl ester of cyclic LPA formed as a result of the CH_2N_2 treatment. The *sn*-2,3-G3P cyclic structure resulted from an internal transesterification reaction, presumably attack of the *sn*-2 hydroxyl group on a methyl phosphate group catalyzed by traces of acid carried over from the phospholipid isolation procedure. These data will be described in detail elsewhere (JE Cronan, PG Roughan, unpublished data). In this work the two LPA spots were quantitated together.

The positional specificity of the acylation of [2-^{3}H]G3P to monoacyl-G3P was analyzed by a modification of the methods described by Bertrams and Heinz (3). A Hepes buffer was used so that hydrolysis to monoglyceride could be done in the G3P acyltransferase assay mixture. Chromatographic system D of Thomas *et al.* (30) was used to separate the isomeric monoglycerides.

G3P concentrations were determined spectrophotometrically by G3P dehydrogenase assay of $HClO_4$ extracts of purified chloroplasts (14).

RESULTS

Characterization of G3P Acylation by Amaranthus Chloroplast Extracts. Lysates of amaranthus chloroplasts readily acylated G3P to a mixture of LPA and PA. Ultracentrifugation supernatants (stroma) formed LPA almost exclusively, whereas uncentrifuged lysates or lysates centrifuged at lower speeds synthesized more PA and less LPA, the proportion of PA being roughly proportional to the amount of membrane material present. Thus in common with the situation in pea and spinach chloroplasts (1, 12), G3P acylation is catalyzed by a soluble protein whereas the acylation of LPA involves a membrane bound enzyme (see below). The G3P acyltransferase had a K_m of 60 to 100 μM for G3P when assayed with palmitoyl-CoA, palmitoyl-ACP or an equimolar mixture of palmitoyl CoA and oleoyl-CoA (data not shown).

The G3P acyltransferase activity of amaranthus was very unstable. In contrast to the pea and spinach enzyme (1, 3, 12) >90% of the amaranthus activity was lost during 16 h of storage at 4 or −20°C. We were unable to stabilize these extracts by increasing the protein concentration or by addition of glycerol, manipulations known to stabilize the other plant G3P acyltransferases (1, 3, 12). Addition of lyotrophic salts (K-phosphate, $(NH_4)_2SO_4$ either with or without glycerol) also failed to increase stability of the amaranthus enzyme. Due to this instability we were unable to purify the enzyme and all experiments were performed with extracts obtained from chloroplasts isolated on the day of the experiment. The same assay conditions were used to study acylation by the amaranthus, pea, and spinach stromal extracts in order to facilitate direct comparisons. Since high quality chloroplasts are most conveniently prepared in relatively small quantities (1–2 mg of Chl per preparation), the amount of protein available precluded use of ^{14}C labeled substrates. Most work done with ^{32}P-labeled G3P to provide the needed sensitivity and to facilitate autoradiography of chromatograms.

The ultracentrifuged stromal preparations were essentially free of interfering activities. The amaranthus preparations (even those prepared by low speed centrifugation) were unable to degrade any of the substrates (G3P, oleoyl and palmitoyl thioesters of CoA or ACP) or the products of the reaction (LPA and PA) during the incubation periods tested (up to 60 min).

Specificity of G3P Acylation. Palmitoyl-CoA was the most active acyl donor for the amaranthus G3P acyltransferase (Table

Table I. *G3P Acyltransferase Activity of Stromal Preparations*

Preparation	Acyl Donor[a]					
	Palmitoyl-CoA[b] + oleoyl-CoA	Palmitoyl-CoA	Oleoyl-CoA	Stearoyl-CoA	Palmitoyl-ACP	Oleoyl-ACP
	units/mg protein[c]					
A. lividus	129.6	148.2	44.8	25.6	23.6	1.6
S. oleracea	62.7	16.6	112.4	8.1	2.9	0.02
P. sativum	1150.0	708.1	1288.3	324.7	65.3	10.1

[a] CoA and ACP donors were present at final concentrations of 90 and 20 μM, respectively. [b] An equimolar mixture of palmitoyl-CoA and oleoyl-CoA was used. [c] A unit of activity is 1 pmol of G3P incorporated/min.

I). Oleoyl CoA was less active, giving G3P acylation rates 30 to 90% that of palmitoyl-CoA. Stearoyl-CoA was a poor acyl donor, a result consistent with the scarcity of stearic acid in amaranthus chloroplast lipids (22, 23). Acyl-ACP substrates were also utilized by the amaranthus enzyme but the rates were lower than found with acyl-CoA donors (Table I). At comparable concentrations (2–10 μM) palmitoyl-CoA and palmitoyl-ACP were about equally active whereas oleoyl-ACP was much less active. This situation (observed previously for the pea and spinach enzymes (3, 8)) may reflect differences between *E. coli* ACP and the plant ACPs. No incorporation was found in the absence of an added acyl donor.

In contrast to amaranthus, oleoyl CoA was a better acyl donor than palmitoyl-CoA for the spinach and pea enzymes (Table I). Indeed, spinach preparations gave oleoyl-CoA rates that were 7- to 9-fold greater than the palmitoyl-CoA rates.

We examined the positional acylation of G3P to LPA catalyzed by the amaranthus extracts. The LPA formed was the 1-monoacyl species; <6% 2-mono-G3P was present (data not shown). This result was found with each of the acyl donors of Table I as well as with an equimolar mixture of the three CoA esters. The pea and spinach enzymes also specifically acylate position 1 of G3P (3, 8).

Selectivity of G3P Acylation. Bertrams and Heinz (3) and Frentzen *et al.* (8) reported that the pea and spinach G3P acyltransferases select oleoyl-thioesters from mixtures of palmitoyl and stearoyl donors. We have repeated these experiments using stromal preparations and our results are in good agreement with those obtained with purified enzymes by the earlier workers (Fig. 1B). In contrast, parallel experiments with amaranthus stromal extracts gave no evidence for selective utilization of oleoyl donors. When presented with mixtures of oleoyl-CoA and palmitoyl-CoA or of oleoyl-ACP and palmitoyl-ACP, the donors were incorporated into 1-monacyl-G3P in the same ratio as that supplied in the substrate mixture (Fig. 1A). However, the amaranthus G3P acyltransferase selected against stearoyl-CoA (Fig. 2), a result consistent with the lipid compositions observed *in vivo* (22, 23). The selectivity of acylation of G3P with mixtures of palmitoyl-CoA and oleoyl-CoA was unaffected by varying the G3P concentration in the assay from 25 to 500 μM (data not shown). It should be noted that the pea and spinach enzyme failed to select against stearoyl thioesters when presented together with palmitoyl thioesters (Fig. 2) (3, 8), a result inconsistent with the lipids synthesized *in vivo*.

Acylation of 1-Monoacyl-G3P. The formation of PA was somewhat variable among amaranthus extracts depending on the preparation. However, in common with the results obtained with pea and spinach chloroplast fractions (3, 8, 12), 1-monoacyl-G3P acyltransferase was membrane bound and had a strong (5–10-fold) selectivity for palmitoyl-CoA over oleoyl-CoA (data not shown). We also examined the selectivity of the enzyme for the 1-monoacyl-G3P substrate (Fig. 3). Amaranthus stromal fractions derived by ultracentrifugation were incubated with ^{32}P-labeled G3P and an equimolar mixture of palmitoyl-CoA and oleoyl-CoA. Following LPA formation incorporation of ^{32}P-labeled G3P into LPA was blocked by addition of a 20-fold excess of nonradioactive G3P. An amaranthus envelope fraction was then added to catalyze the acylation of LPA to PA. Fractions were taken at various times after envelope addition and the LPA species were resolved by $AgNO_3$ TLC or reverse phase TLC. No

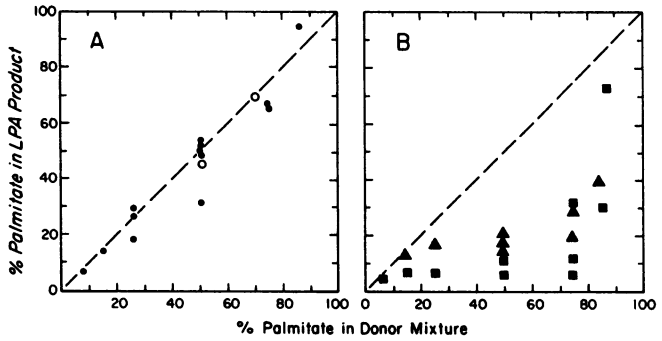

FIG. 1. Selectivity in the acylation of G3P with mixtures of palmitoyl and oleoyl acyl donors. The reaction products were separated and analyzed as described in Materials and Methods. The symbols are amaranthus stroma (●, acyl-CoA; ○, acyl-ACP) in panel A whereas the results of acylation with acyl-CoA mixtures catalyzed by the pea and spinach stroma (panel B) are denoted by ▲ and ■, respectively. The dashed line is that expected if no acylation sensitivity exists. The total concentrations of CoA and ACP donors were 90 and 10 μM, respectively.

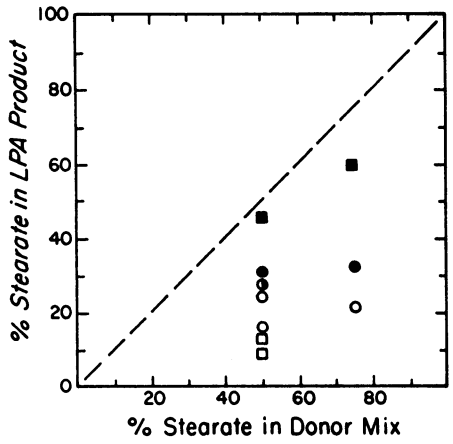

FIG. 2. Selectivity in the acylation of G3P with mixtures containing stearoyl-CoA. Results obtained with amaranthus enzyme using mixtures of stearoyl-CoA with either oleoyl-CoA or palmitoyl-CoA are denoted by ● and ○, respectively. For comparison, data obtained with the spinach enzyme and the same mixtures are shown (oleoyl-CoA mixtures □; palmitoyl-CoA ■). The dashed line is that expected in the absence of selectivity. The total acyl donor concentration was 90 μM.

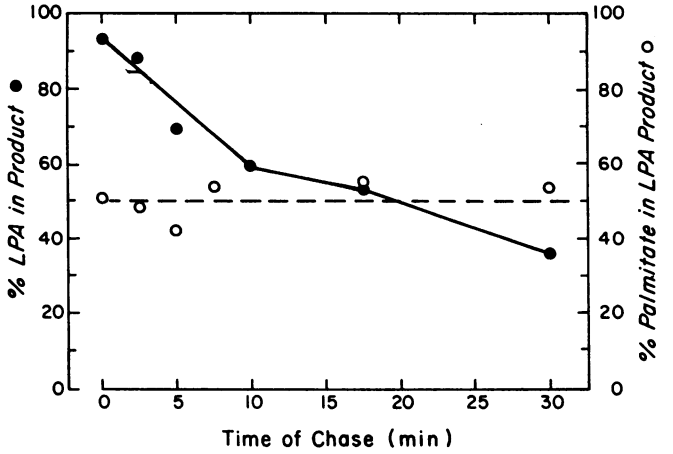

FIG. 3. Selectivity in the acylation of 1-monoacyl G3P. An amaranthus stromal extract (20 μl, 420 μg protein) obtained by ultracentrifugation was incubated with an equimolar mixture of oleoyl-CoA and palmitoyl-CoA (total concentration of 90 μM) and ^{32}P-labeled G3P as described in "Materials and Methods." After a 15 min incubation, a 20-fold molar excess in nonradioactive G3P was added together with 10 μl of a yellow (largely thalikoid-free) envelope preparation (3.1 mg protein) derived by ultracentrifugation following low speed centrifugation of lysed chloroplasts. Samples were then taken onto filter disks at the same times shown and the lipids resolved as described in "Materials and Methods." Symbols: ●, percent LPA remaining during conversion to PA; ○, percent palmitate in the unreacted LPA.

preferential utilization of 1-monooleoyl-G3P or 1-monopalmitoyl-G3P for PA synthesis was observed (Fig. 3). Likewise a similar experiment showed that 1-monooleoyl-G3P and 1-monostearoyl-G3P were acylated at the same rate (data not shown). However, 1-monopalmitoyl-G3P was converted to PA at twice the rate of 1-monostearoyl-G3P when a mixture of these LPAs was acylated (data not shown).

Chloroplast G3P Levels. Bertrams and Heinz (3) and Frentzen *et al.* (8) reported that the acylation selectivity of the pea and spinach G3P acyltransferase was affected by the concentration of G3P present in the assay, somewhat greater specificity being observed at lower G3P concentrations. As mentioned above, no such effect was seen with the amaranthus stroma. To put these results in a physiological context we determined the stromal G3P concentration of amaranthus chloroplasts using G3P dehydrogenase. The G3P concentrations found (21–29 nmol/mg Chl) were about 7-fold greater than the values reported in spinach chloroplasts (25) and suggest a stroma G3P concentration of 450 to 620 μM assuming 47 μl/mg Chl for the stromal volume. Thus the amaranthus G3P acyltransferase appears to be saturated with G3P *in vitro*.

DISCUSSION

Our most important finding is that the amaranthus G3P acyltransferase failed to discriminate between oleoyl and palmitoyl donors when a mixture of the two acyl thioester substrates was utilized. Roughan (24) has shown that the pool of acyl donors synthesized *in vitro* by amaranthus chlorplasts (during a 10 min incubation) consists of palmitoyl-ACP, oleoyl-ACP, and stearoyl-ACP in proportion of 0.8:1:1. This mixture of acyl donors coupled with the properties of the G3P acyltransferase and the 1-monoacyl-G3P acyltransferase reported in this paper indicate that the glycerolipids synthesized by amaranthus chloroplasts should contain a high proportion of disaturated species, the result obtained upon direct analysis (22, 23). Spinach (27) and pea (PG Roughan, unpublished data) chloroplasts synthesize mixtures of acyl-ACP donors quite similar to that of amaranthus but the preferential utilization of oleoyl donors (3, 8) accounts for the much lower levels of disaturated glycerolipids synthesized by these chloroplasts (22). Therefore, in all three plants the pattern of substrate utilization by the acyltransferase adequately accounts for the saturated to unsaturated ratios of the lipids synthesized by the chloroplasts. The utilization is essentially identical for acyl-CoA and acyl-ACP thioesters. However, the substrates believed to function *in vivo*, acylated derivatives of the plant ACPs, have not yet been tested. Our acyl-CoA acylation experiments were done using concentrations and conditions recently demonstrated (18) to give monomolecular (rather than micellar) solutions of these amphiphiles. However, similar selectivities were obtained at acyl-CoA concentrations above the critical micelle concentrations.

It should be noted however that interpretation of the data on the spinach and pea G3P acyltransferases is not completely straightforward. For example neither enzyme selects strongly against stearoyl donors when a mixture of stearoyl and palmitoyl donors is presented. Thus, the scarcity of stearate in the lipids of pea and spinach may be due to another factor, for example preferential utilization for desaturation to oleate. Moreover, the chloroplast G3P acyltransferases of these plants seem kinetically complex. As demonstrated (but not discussed) by Frentzen *et al.* (8), oleoyl-ACP is utilized for synthesis of 1-acyl G3P at a much lower maximal velocity than are palmitoyl-ACP and stearoyl-ACP when each is provided alone (see also Table I). However, when presented with mixtures of oleoyl-ACP and the other acyl-ACPs (at saturating concentrations of each donor and of G3P), oleoyl groups are selectively incorporated into 1-acyl G3P. About 90% of the product is oleoyl-G3P when oleoyl-ACP comprises only 50% of the acyl donor pool (8). These data argue that oleoyl-ACP becomes a more active acyl donor in the presence of palmitoyl-ACP or stearoyl-ACP and thus imply an allosteric mechanism for the enzymes of pea and spinach.

There is a strong correlation between the level of disaturated PG in the chloroplast lipids of a plant and chilling sensitivity of the plant. Our results suggest that altering the ratio of oleoyl-ACP to palmitoyl-ACP synthesized by the chloroplast should directly affect the content of chlorplast disaturated PG. The results of various manipulations of the spinach chloroplast fatty acid synthase system *in vitro* (26) suggest several possible methods that could alter the oleoyl-ACP:palmitoyl-ACP ratio. These suggestions should be testable *in vivo* due to the recent advances in genetic engineering of chloroplast proteins and thus could allow assessment of the hypothesized relationship between chloroplast PG saturation and chilling sensitivity.

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