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CHARACTERIZATION OF GALACTOLIPID SYNTHESIS IN PEA ROOT PLASTIDS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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ABSTRACT

The capacity of pea root plastids for galactolipid synthesis was investigated utilizing radiolabelled acetate and UDP-galactose. Galactolipid biosynthesis was completely dependent on an exogenous supply of UDP-galactose. UDP-galactose stimulated both total lipid biosynthesis from acetate and the proportion of radioactivity accumulated in monogalactosyldiacylglycerol (MGDG). The proportion of MGDG synthesized was saturated at 30μM UDP-galactose and represented approximately 30% of the total lipid radioactivity after a one hour incubation. However, total lipid biosynthesis continued to increase with concentrations of UDP-galactose up to 75μM while the proportion of radioactivity in MGDG remained at 30%. MGDG biosynthesis was always accompanied by a corresponding decrease in the amount of diacylglycerol (DAG) accumulated. Digalactosyldiacylglycerol (DGDG) synthesis was not routinely observed in these experiments. These results suggest that the *in vitro* pathway for MGDG synthesis in the root plastids of pea (an 18:3 plant) is similar to 16:3 plants (FFA's→PA→DAG→MGDG). The endogenous lipids, consistent with the thought of pea as an 18:3 plant, contained 80% C_{18} in the fatty acids of MGDG, DGDG, TG and PC. However, in labelled acetate experiments palmitate was

the predominately labelled fatty acid in all lipids except PC (where 80% was 18:1). The precursors PA and DAG had ratios of 16:0, 18:0, and 18:1 similar to that of MGDG. 70-80% of the label was associated with the *sn-2* position of glycerolipids. The cofactors required for fatty acid synthesis were generally not as required for galactolipid synthesis. The results suggest that galactolipid synthesis relies primarily on endogenous DAG and only partly involves *de novo* fatty acid synthesis. These results indicate that pea root plastids are capable of MGDG synthesis in amounts comparable to that found in photosynthetic tissues and organelles, and indicate that pea root plastids retain their ability for galactolipid biosynthesis after tissue differentiation even though they are nonphotosynthetic, nongreening plastids from an 18:3 plant.

RESUME

La capacité de synthèse des galactolipides par les plastes de racine de pois a été étudiée à l'aide d'acétate et d'UDP-galactose marqués radioactivement. La biosynthèse des galactolipides a été totalement dépendante de l'apport exogène d'UDP-galactose. L'UDP-galactose a stimulé la production des lipides totaux synthétisés à partir de l'acétate, ainsi que l'incorporation de radioactivité dans le monogalactosyldiacylglycérol (MGDG). La synthèse de MGDG est parvenue à saturation lorsque l'apport d'UDP-galactose atteignait une concentration de 30μM, le MGDG représentant approximativement 30% de la radioactivité incorporée dans les lipides totaux après une incubation d'une heure. Cependant, la biosynthèse des lipides totaux s'est poursuivie jusqu'à ce que la concentration d'UDP-galactose atteigne une valeur de 75μM, la proportion de radioactivité incorporée dans le MGDG restant de 30% . La biosynthèse de MGDG a toujours été accompagnée par une diminution parallèle du pool de diacylglycerol (DAG) nouvellement formé. La biosynthèse de digalactosyldiacylglycerol (DGDG) n'a pratiquement jamais été observée au cours de ces expériences. Ces résultats suggèrent que, *in vitro*, la voie de synthèse du MGDG dans les plastes de racine de pois (plante en 18:3) est similaire à celle des plantes en 16:3 (Acides gras libres → Acide phosphatidique (AP) → DAG →

MGDG). En accord avec l'appartenance du pois au groupe des plantes en 18:3, les lipides endogènes présentaient 80% de C_{18} dans les acides gras du MGDG, du DGDG, du triacylglycerol et de la phosphatidylcholine. Cependant, lors des expériences utilisant l'acétate marqué, le palmitate a été l'acide gras marqué prédominant dans tous les lipides, excepté dans la phosphatidylcholine (80% de 18:1). Les précurseurs AP et DAG contenaient des quantités relatives de 16:0, 18:0 et 18:1 similaires à celles du MGDG. Une proportion de 70 à 80% du marquage était associée à la position *sn-2* des glycérolipides. Les cofacteurs, indispensables à la synthèse des acides gras, ne l'étaient en général pas autant pour la synthèse des galactolipides. Ces résultats suggèrent que la synthèse des galactolipides se fait principalement dépend au du DAG endogène et ne requiert que partiellement une synthèse *de novo* des acides gras. Ceci indique que les plastes de racine de pois sont capables de synthétiser le MGDG en quantité comparable à celle trouvée dans les tissus et organites photosynthétiques. Bien que les plastes de racine de pois, plante en 18:3, ne soient plus photosynthétiques et chlorophylliens après la différenciation tissulaire, ils conservent leur capacité de biosynthèse des galactolipides.

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ACP:	Acyl carrier protein
ADP:	Adenosine-5′-diphosphate
ASG:	Acylsterol glycerol
ATP:	Adenosine-5′-triphosphate
CDP:	Cytidine-5′-diphosphate
CMP:	Cytidine-5′-monophosphate
CTP:	Cytidine-5′-triphosphate
CoA:	Coenzyme A
DAG:	Diacylglycerol
DGDG:	Digalactoslydiacylglycerol
ER:	Endoplasmic reticulum
FFA:	Free fatty acid
G3P:	glycerol-3-phosphate
GLC:	Gas liquid chromatography
LPA:	Lysophosphatidic acid
LPC:	Lysophosphatidylcholine
MAG:	Monoacylglycerol
MGDG:	Monogalactoslydiacylglycerol
NADH:	Nicotinamide adenine dinucleotide (reduced form)
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form)
PA:	Phosphatidic acid
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PG:	Phosphatidylglycerol
PI:	Phosphatidylinositol
PS:	Phosphatidylserine
SE:	Sterol esters
SL:	Sulfolipid
SQDG:	Sulfoquinovosyldiacylglycerol
TAG:	Triacylglycerol
TLC:	Thin layer chromatography
UDP:	Uridine-5′-diphosphate
18:1:	Specific fatty acids with the number of carbons followed by the number of double bonds.

INTRODUCTION

Lipids are essential for many biological activities through their importance in membranes, carbon and energy storage and regulation of development. Lipids, and specifically galactolipids, have been identified as important in thylakoid structure (Webb and Green, 1991), chilling and temperature stress tolerance (Kaniuga and Gemel, 1984; Quinn and Williams, 1983), senescence (Dalgarn *et al.*, 1979), ozone injury (Sakaki *et al.*, 1990), herbicide activity (Lem and Williams, 1983), and the development of new and improved plant oils (Somerville and Browse, 1991; Ohlrogge *et al.* 1991). The importance of photosynthesis and the economic significance of oilseeds has promoted much of the research on galactolipids to focus in these areas. Except for the identification of various lipids in roots (Sparace and Kleppinger-Sparace,1993), little work has focused on lipid synthesis within roots, especially galactolipid synthesis. This study is aimed at characterizing galactolipid synthesis in roots as part of a larger study to characterize the mode of regulation of higher plant lipid metabolism in non-photosynthetic tissues.

Pea root leucoplast are used in this study for a number of reasons. Among others, they are a useful tool for the study

of lipid metabolism in roots (Sparace and Kleppinger-Sparace, 1993). Although less specialized than chloroplasts and oilseed plastids, they are also actively and independently involved in fatty acid and glycerolipid biosynthesis (Stahl and Sparace, 1991; Xue, 1993). Finally and most importantly, although plastids are considered to be the site of galactolipid biosynthesis in plants (Joyard *et al.*, 1993), galactolipid biosynthesis has never been described in root plastids.

The working hypothesis of this research is that galactolipid synthesis in pea root plastids is similar to that of chloroplasts. With this hypothesis, the general objective of this research is to characterize galactolipid biosynthesis in these plastids. More specific objectives are the following:

1. Determine the endogenous fatty acid and glycerolipid composition of pea root plastids.
2. Determine the optimum *in vitro* cofactors and substrate requirements for galactolipid biosynthesis from acetate and UDP-galactose.
3. Determine the radioactive fatty acid composition and positional distribution in acetate-labelled galactolipid in comparison to other glycerolipids.

LITERATURE REVIEW

Biological Importance of Galactolipids

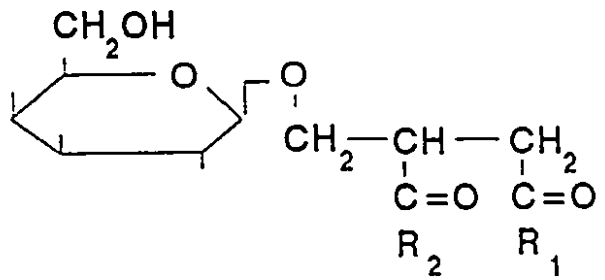
Galactolipids are polar neutral lipids containing one or more galactose residues at the *sn-3* position of diacylglycerol. They are the major membrane constituents of both thylakoids and envelope membranes in chloroplasts. As such, they are the principle component of the fluid bilayer where their asymmetry is important in the activity of certain enzymes as well as the structural stability of these important membranes (Douce and Joyard, 1980; Hincha *et al.*, 1993; Grant and Peters, 1984).

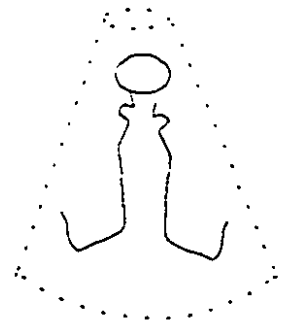
Galactolipids, primarily monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol(DGDG), represent 80% of the total polar lipids in chloroplasts. In photosynthetic tissue they largely reflect the thylakoid lipid composition, enriched in MGDG. This fact leads MGDG to be acclaimed the most abundant polar lipid in the world (Gounaris *et al.*, 1986). In nonphotosynthetic tissue the primary galactolipid-containing membrane is the envelope membrane. Here there is a greater ratio of DGDG to MGDG and a greater ratio of phospholipid to galactolipid (for reviews see Douce and Joyard, 1980; Harwood, 1989; Browse and Somerville, 1991; Joyard *et al.*, 1993; Sparace and Kleppinger-Sparace, 1993).

Galactolipids, particularly MGDG are extremely important in thylakoid biogenesis and photosynthesis in chloroplasts. Upon light induction, the production of prolamellar bodies of proplastids is accompanied by an increase in MGDG synthesis (Fishwich and Wright, 1980). MGDG has a relatively small polar head and bulky hydrophobic acyl chains. These characteristics give this lipid a 3-dimensional cone shape (figure 1). Further, this lipid cannot independently form bilayer phases (Gounaris *et al.*, 1986). Its structure is thought to be important in the formation of the thylakoids by stabilizing the concave curvature required for stacking, and helping to package the photosynthetic protein complexes of the thylakoids (Murphy, 1982). Specifically chlorophyll-protein complexes #43 and #47 of the photosystem II core are enriched with MGDG and contain high levels of saturated fatty acids (Tremolieres *et al.*, 1994). MGDG is also thought to be important for the insertion of the protein ferredoxin into the thylakoids (Chupin *et al.*, 1994), and is required for the associations of chlorophylls a and b in photosystem II (Gounaris *et al.*, 1986).

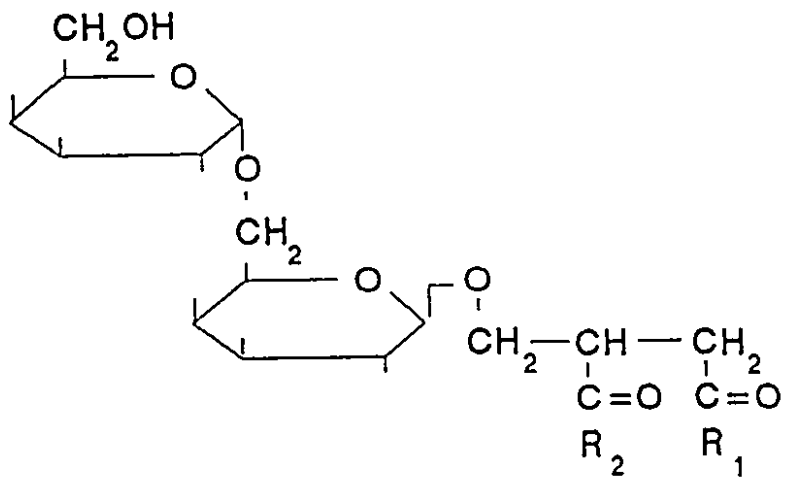
In contrast to MGDG, DGDG has a wedge or cylindrical 3-dimensional shape (figure 1). This shape is thought to help stabilize the convex curvature of the thylakoids (Murphy, 1982). It also provides a protective ring for the light

MGDG





DGDG



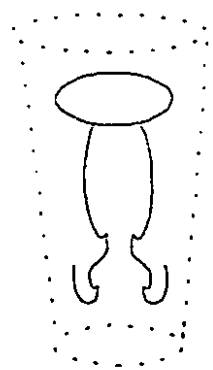


Figure 1. The chemical structure of MGDG and DGDG including the schematic representation of their shapes. R_1 and R_2 indicate fatty acids esterified to the *sn-1* and *sn-2* positions, respectively.

harvesting complex II (Nussberger *et al.*, 1993) and interacts with MGDG to form the planar bilayer of the thylakoids (Fuks and Homble, 1994). The galactolipids are known for their importance as diffusion pathways of ions within the thylakoids (Webb and Green, 1991).

Galactolipids have been implicated in a number of environmental or stress-related physiological phenomena in plants. The activity of herbicides, particularly the pyridazinones, affect chloroplast membrane stability by inhibiting the desaturation of MGDG through the reduction of linolenic acid to linoleic acid (Lem and Williams, 1983). Similarly triazine resistant *Amaranthus* is known to contain higher amounts of galactolipid and linolenic acid compared to the susceptible variety (Dominquez *et al.*, 1994).

In addition to herbicide effects, atmospheric pollutants can also affect chloroplast galactolipids. Ozone caused a decrease in the content of MGDG and results in the destruction of membrane lipids by acting on the double bonds of unsaturated fatty acids (Sakaki *et al.*, 1990). As MGDG and linolenic acid decrease, TAG and linoleic acid increase. During this process, MGDG is converted to FFA and DAG which are subsequently used for TAG formation (Sakaki *et al.*, 1994: Rais *et al.*, 1993). Similarly, studies of Norway spruce with acidic misting and ozone showed decreases in the

unsaturation of both C_{16} and C_{18} fatty acids of MGDG. These changes occurred as a result of altered desaturase activity on the PC in the ER. Usually a delta-6 double bond is located on the C_{18} fatty acids produced from PC but is now changed to the differently shaped delta-5 in ozone-treated spruce needles (Wellburn *et al.*, 1994).

There has been a substantial amount of research on the relationship of MGDG and cold hardening. As plants are acclimatized to the cold there is an increase in MGDG and 18:3 (Saczynska *et al.*, 1993; Alberdi *et al.*, 1993). However, cold sensitive plants likewise have a greater amount of FFA and galactolipase activity (Kaniuga and Gemel, 1984). Membranes are apparently the primary site of injury. As the temperature drops there can be a change in the membrane state from liquid-crystalline to solid gel. This causes cracks and channels which increase permeability leading to solute influx and ultimately rupture of the membrane (Lyons *et al.*, 1979; Quinn and Williams, 1983; Webb and Green, 1991). Increasing the level of unsaturation during acclimation can decrease the stress to the membrane by lowering the temperature of phase transition and preventing changes in the hydrophobic matrix of the membrane. Further, changes in this matrix can also affect membrane bound proteins (Alberdi and Corcuera, 1991). Bakaltcheva *et al.* (1994) have shown that the solute influx

in freeze-thaw damage also directly affects the damage to thylakoids by causing a release of plastocyanin and thus the inactivation of light driven ATP synthesis in chloroplasts. Finally, DGDG may also play a role in cryoprotection of thylakoid membranes by creating binding surfaces for galactose specific lectins. These lectins inhibit increased solute permeability of chloroplast membranes during freezing and thawing (Grant and Peters, 1984; Hincha *et al.*, 1993).

Glycerolipid Composition and Biosynthetic Capacities of Plastids

As mentioned earlier, galactolipids comprise a major proportion of the total lipids of chloroplasts. These lipids are also important constituents of nonphotosynthetic plastids such as potato tuber amyloplasts (Fishwich and Wright, 1980), daffodil chromoplasts (Kleinig and Liedvogel, 1978), cauliflower bud plastids (Journet and Douce, 1985), and beet chromoplasts (Wintermans, 1960). The amount of galactolipid in these plastids ranges from 14% MGDG in potato tuber amyloplasts to 63% in daffodil chromoplasts. The presence of DGDG varies from 18% in daffodil chromoplasts to 45% in potato tuber amyloplasts. In addition to galactolipids plastid membranes also contain varying amounts of primarily sulfolipid (SQDG), PG, and PC (Sparace and Kleppinger-Sparace, 1993). Similarly, plastids show varying capacities to synthesize their component

glycerolipids. Overall, the precise lipid compositions and biosynthetic capacities of the various types of plastids is apparently related to the nature of the plastid and the tissue from which it is derived (Sparace and Kleppinger-Sparace, 1993).

Pea root plastids are emerging as a model for lipid metabolism in a nonphotosynthetic root plastid. These plastids have been characterized for their optimum *in vitro* requirements and capacities for fatty acid and glycerolipid biosynthesis (Kleppinger-Sparace *et al.*, 1992; Stahl and Sparace, 1991; Xue, 1993). In one preliminary experiment Xue (1993) also determined the glycerolipid composition of pea root plastids. Her tentative results, never repeated, suggested that these plastids contain a mole percent of 5.9% MGDG and 6.7% DGDG and that the galactolipids contained primarily 16-22% linoleic acid (18:2) and 58-65% linolenic acid (18:3).

Galactolipid Structure

Galactolipids were first isolated from wheat flour by Carter *et al.* (1956). Figure 1 illustrates the structure of MGDG and DGDG. Carter first defined galactolipid structure as 2,3-diglycerides with a carbohydrate (galactose) moiety attached to the C-1 position of glycerol. According to the IUAPCA-IUB Commission of Biochemical Nomenclature MGDG

refers to 1,2-diacyl-3-O-(ß-D-galactopyranosyl)-sn-glycerol while DGDG is 1,2-diacyl-3-O-(α-D-galactopyranosyl-(1-6)-O-ß-D-galactopyranosyl)sn-glycerol (Douce and Joyard, 1980).

Galactolipids contain various amounts of the typical fatty acids found in plants, particularly palmitic, stearic, oleic, linoleic, and linolenic acids. However galactolipids are characteristically enriched in one of two different polyunsaturated trienoic acids, palmitolinolenic (16:3) and linolenic acid (18:3). The type of these trienoic acids found in the galactolipids is species specific (Jamieson and Reid, 1971) and has given rise to the trivial distinctions of "16:3 plants" vs. "18:3 plants" (Roughan and Slack, 1984).

Prokaryotic vs. Eukaryotic Pathways for Glycerolipid Assembly

There are two generally recognized pathways of glycerolipid biosynthesis in plants. Those synthesized external to the plastid, primarily in the ER, are termed eukaryotic. The eukaryotic pathway produces a DAG (via PC) with two 18 carbon fatty acid chains. The prokaryotic pathway, occurring within the plastid, produces lipids with 16 carbon (C_{16}) fatty acids at the *sn-2* position and 18 carbon (C_{18}) fatty acids in the *sn-1* position.

The so-called "16:3 plants" have a higher plastidic PA phosphatase activity and can therefore generate sufficient DAG to support galactolipid biosynthesis (see figure 2.0). In 18:3 plants a low PA phosphatase activity (Heinz and Roughan, 1983) means they cannot generate this DAG and therefore must rely on the eukaryotic pathway. 18:3 plants therefore contain only small amounts of galactolipids with 16 carbon fatty acids but do contain PG with such fatty acids as generated from PA. In the eukaryotic pathway for galactolipid synthesis there is a reversal of an envelope-bound CDP-choline:glyceride transferase which releases the DAG moiety of PC for galactosylation (Roughan and Slack, 1984). The DAG precursors in *Vicia faba* leaves (an 18:3 plant) contain highly unsaturated fatty acids, particularly C18:2 and C18:3 (Lem and Williams, 1983; Williams *et al.*, 1988). Examples of 18:3 plants include; *Pisium sativum* (used here), *Vicia faba, Avena sativa, Zea mays* and *Medicago sativa* while 16:3 plants include: *Arabidopsis thaliana, Spinacia oleracea, Brassica napus* and *Nicotiana sylvestris* (Heemskerk *et al.*, 1990; Williams *et al.*, 1988).

Biosynthesis of MGDG and DGDG

Glycerolipid assembly in plastids occurs on the envelope. The process requires a supply of fatty acids which are used for the acylation of glycerol-3-phosphate. These fatty acids are provided in the form of acyl-ACP's (typically

16:0-ACP and 18:1-ACP) by the process of *de novo* fatty acid biosynthesis which occurs in plastids (Stumpf, 1984). In contrast, glycerol-3-phosphate originates both in the cytosol and the plastid stroma. It is produced by DHAP reductase from DHAP derived from either the Calvin cycle or glycolytic metabolism.

The process of galactolipid biosynthesis is illustrated in figure 2. As shown, glycerol-3-phosphate is acylated to form LPA as an acyl-carrier protein donates 18:1 to the *sn-1* position. This reaction is catalyzed by glycerol-3-phosphate acyltransferase. PA is then produced from LPA in the reaction where 1-acylglycerol-3-phosphate acyltransferase adds a 16:0 to the *sn-2* position of L-PA. This latter reaction is characteristic of a 16:3 plant using the "prokaryotic" (plastidic) pathway for glycerolipid assemble. In comparison the "eukaryotic" pathway would transfer 18-carbon fatty acid chains at both the *sn-1* and *sn-2* positions) (Joyard *et al.*, 1993). Phosphatidate phosphatase removes the phosphate of PA to form DAG. Alternatively, in the eukaryotic pathway, DAG is thought to originate from cytosolic PC by a reversal of CDP-choline:glyceride transferase (Roughan and Slack, 1984). MGDG from either prokaryotic or eukaryotic DAG is finally produced by the addition of galactose from UDP-galactose to DAG, via monogalactosyldiacylglycerol synthase. DGDG is

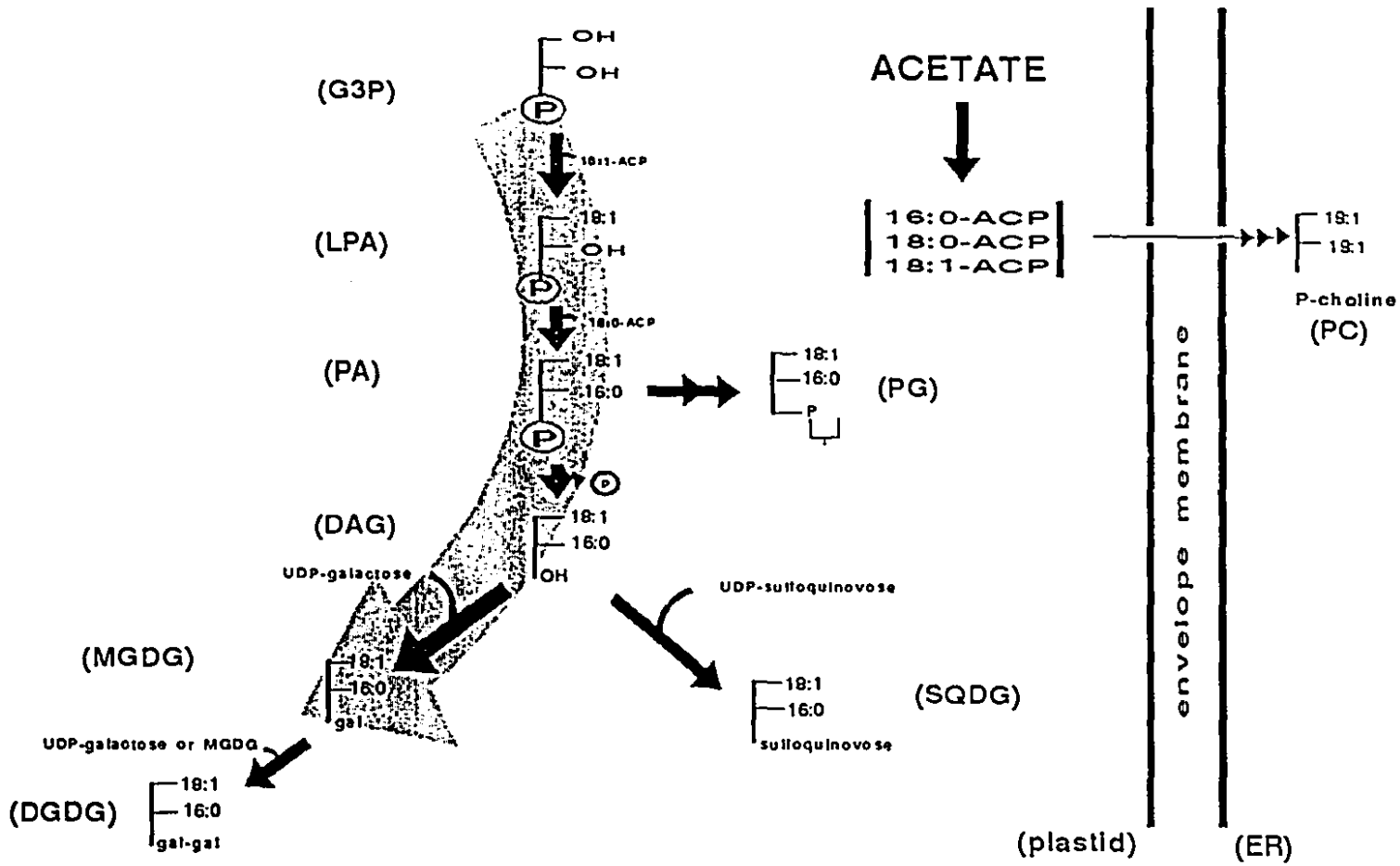


Figure 2. Pathway for galactolipid synthesis in plastids.

then produced either via galactosylation by the addition of another galactose from UDP-galactose to MGDG or transgalactosylation by transferring the galactose from a second MGDG to form DGDG + diacylglycerol. Eventually desaturases can then operate on either the PC outside the plastid or the galactolipids within the plastids by adding double bonds to the fatty acid chains (Douce and Joyard, 1980; Roughan and Slack, 1984; Joyard *et al.*, 1993).

As the galactolipids are produced in the chloroplast envelope there is a preferential export of MGDG over DGDG to the thylakoids. In intact plastids the ratio of MGDG/DGDG is 7 but in thylakoids it is 18. The outer thylakoid membrane alone is 64% MGDG and 15% DGDG (Rawyler *et al.*,1992). The ratio of MGDG:DGDG increases with grana production from 1:1 to 2:1 (Leech *et al.*, 1973).

Figure 2 also illustrates the production of SQDG from DAG. With the addition of UDP-sulfoquinovose, the activity of SQDG synthase can compete with MGDG synthase for DAG molecules (Kleppinger-Sparace *et al.*, 1985; Joyard *et al.*, 1993). However, SQDG synthase is known to have a pH optimum lower than MGDG synthase and has a greater preference for 16:0/16:0-DAG than MGDG synthase.

Origin of UDP-galactose in Relation to Galactolipid Synthesis

The UDP-galactose required for galactolipid synthesis is synthesized from UDP-glucose by UDP-glucose epimerase. The enzymes involved in UDP-glucose and UDP-galactose synthesis, are located in the cytosol (Konigs and Heinz, 1974). However, MGDG synthase (1,2-diacylglycerol 3-B-galactosyltransferase) is a plastid envelope enzyme (Douce, 1974) located on the inner envelope membrane which is impermeable to UDP-galactose. Therefore, MGDG synthesis most likely occurs on the outer surface of the inner envelope membrane (Joyard *et al.*, 1993), although in pea it was demonstrated in the outer envelope (Cline and Keegstra, 1983). Miquel and Dubacq (1992) found galactolipid synthesis was higher in the inner membrane but present in both the inner and outer envelope membrane of pea chloroplasts. Galactolipid: galactolipid galactosyltransferase is also located on the outer envelope membrane (Heemskerk *et al.*, 1986). Envelope membranes can thus synthesize some MGDG without UDP-galactose, where synthesis increases with DAG concentration and DGDG serves as the donator of the galactosyl moiety (Heemskerk *et al.*, 1988).

UDP-Galactose-diglyceride Galactosyltransferase

(MGDG Synthase)

MGDG synthase is a very minor envelope component. It therefore has an extremely high specific activity as it sustains the rates of MGDG synthesis. It has a broad pH optimum above 7.5 and up to 9.0 (Van Besouw and Wintermans, 1979). Stimulation of galactolipid production by this enzyme has been achieved with a high ionic strength, Mg^{2+}, Mn^{2+}, and Ca^{2+} and inhibition with Zn^{2+}, Fe^{2+}, and Cd^{2+} (Heemskerk *et al.*, 1987). The optimum activity of solubilized MGDG synthase requires a negatively charged hydrophobic environment and PG therefore can strongly stimulate its activity (Coves *et al.*, 1988).

The K_m value for UDP-galactose of about 100μM was determined with purified enzyme fractions of MGDG synthase while the amount of UDP-galactose in the cytosol was found to be up to 0.5mM (Coves *et al.*, 1988). MGDG synthase has been found to use all molecular species of DAG. However, it has a specificity for highly unsaturated DAG's (Liedvogel and Kleinig, 1979) and has the lowest activity with 16:0/16:0-DAG. MGDG synthase is a random bireactant system where DAG (at the hydrophobic site) and UDP-galactose (the hydrophillic site) bind randomly to the enzyme and the enzyme is inhibited by UDP (K_i=10μM) (Coves *et al.*, 1988).

Galactolipid:galactolipid Galactosyltransferase

The principal enzyme responsible for DGDG synthesis is the galactolipid:galactolipid galactosyl-transferase located on the cytosolic side of the outer envelope membrane (Van Besouw and Wintermans, 1978: Dorne *et al.*, 1982: Alban *et al.*, 1988). It transfers the galactose moiety from one MGDG to a second MGDG molecule to form DGDG. This is accompanied by a release of DAG. This latter DAG may then go on to form more MGDG via MGDG synthase or TAG with diacylglycerol acyltransferase according to Sakaki *et al.* (1990). Finally unnatural galactolipids with more than two galactose residues can also be produced via transgalactosylation by the galactolipid: galactolipid galactosyltransferase (Heemskerk *et al.*, 1987; Van Besouw and Wintermans, 1978).

The biochemical properties of this enzyme are different from MGDG synthetase. It has an optimum at pH 6.0, requires cations like Mg^{2+} and Ca^{2+} and is inhibited by EDTA, NaF and Zn^{2+}(1mM) (Heemskerk *et al.*, 1987). This may be a result of the specific bond formed, a ß-glycosidic bond in MGDG and an α-glycosidic bond in DGDG. DGDG formation is never stimulated by UDP-galactose but its activity does increase with MGDG concentration (Heemskerk *et al.*, 1990).

This galactosyltransferase enzyme exhibits a strong selection for 18:3/18:3-MGDG as a substrate for galactose transfer (Roughan, 1987). As a result DGDG has been found to accumulate more eukaryotic fatty acids than MGDG. It has been reported in pea seedlings (an 18:3 plant) (Siebertz and Heinz, 1977). Heemskerk *et al.* (1991) found that 16:3 chloroplasts incorporated radiolabelled acetate into prokaryotic galactolipids within two hours. MGDG was produced 10x faster than DGDG. In the *sn-2* position, 16:3 plants exhibit a greater ratio of 16:0 to 16:3 in DGDG, while MGDG had the opposite (Heinz, 1977). Palmitate desaturase does not react with the *sn-2* fatty acid of DGDG.

Purification of Galactolipid-Synthesizing Enzymes

The purification of galactolipid-synthesizing enzymes particularly MGDG synthase has met with limited success. Large quantities of purified envelope membranes are necessary to purify these enzymes. This is difficult because envelope membranes only represent 1-2% of the plastid proteins (and MGDG synthase only 0.1-0.5% of the total envelope proteins). Also, high concentrations of detergents are required for solubilization which inhibit enzyme activity (Joyard *et al.*, 1993). Despite these problems Teucher and Heinz (1991) have partially purified MGDG synthase from chloroplast envelopes. From 10 kg of

spinach leaves they purified 0.02 mg of protein with the enrichment of activity a factor of 250-500. The protein has a molecular weight of 22 kD (in contrast to 19 kD determined by Marechal *et al.*, 1991) and proved to be only a trace component of the mixture of envelope proteins. No selectivity was exhibited by the enzyme regarding galactosylating various DAG's. This is in contrast to results of Coves *et al.* (1988) as discussed earlier. Further work in this area is required for the identification and characterization of these enzymes involved in galactolipid biosynthesis.

MATERIALS AND METHODS

The materials and methods used below are essentially those described by Kleppinger-Sparace *et al.* (1992).

Plant Material

Pea seeds (*Pisium sativum* cv. Improved Laxton's Progress) were imbibed via soaking overnight under running tap water. The seeds were then surface sterilized for 5 minutes with a 5% (v/v) hypochlorite solution. In a sterile laminar flow hood the seeds were rinsed three times with sterile water then placed in a sterile container that contained sterile moistened paper. Seeds were then allowed to germinate for 5-6 days at room temperature(25°C) in the dark.

Plastid Isolation

1-2 cm root tip segments were cut and chilled on ice. The tips were then homogenized for 5 minutes in 2mL of homogenization buffer (50 mM Tricine, 0.33 M Sorbitol, 1.0 mM EDTA (NA_2), 1.0 mM $MgCl_2$ and 0.1% BSA; pH 7.9) per gram fresh weight of root tissue in a chilled mortar and pestle. The intact tissues and cells were removed from the homogenate by filtering through two layers each of 250 and 20 μm nylon mesh.

The filtrate was divided into two centrifuge tubes and centrifuged for 8 minutes at 500*g* (2,000 rpm). The crude plastid pellets were then resuspended in 0.5 mL each of the homogenizing buffer, combined and overlaid onto 5 mLs of centrifugation buffer (50 mM tricine, 0.33 M sorbitol, BSA 0.1% w/v BSA, and 10% v/v Percoll; pH 7.9). The plastids were then purified through the Percoll by centrifugation 4000*g* (6,000 rpm) for 5 minutes. The residual Percoll and BSA in the plastid pellet was removed by resuspension in 5 mL of rinse buffer (1.0mM Bis-Tris propane buffer and 0.33M sorbitol, pH 7.9) followed by centrifugation at 3,000 rpm (1000g) for 5 minutes. The final pellet of purified plastids was then resuspended in 1 mL of rinse buffer. All centrifugations were performed with a Beckman Type 28 rotor. Other workers have shown that these plastids are intact and free of extra plastidic contamination (Xue, 1993; Qi *et. al.*, 1995).

Protein Determination

Plastids left at the end of an experiment were placed in the freezer (-20°C) until their protein concentration could be determined as described by Lowry *et al.* (1951). The plastid protein was first precipitated with trichloroacetic acid (TCA). 200 μL of the plastids was mixed with 1 mL of 10% TCA and allowed to stand for 20 minutes on ice. This was then centrifuged for 10 minutes at maximum speed in a bench-

top centrifuge and the pellet resuspended in 1.0 mL of 0.1N NaOH. A standard curve was prepared using bovine serum albumin (BSA) with concentrations of 0, 10, 25, 50, 75 and 100 μg. The redissolved plastid precipitate was analyzed using 225, 125 and 50 μL of the 1 mL suspension (representing 45, 25 and 10 μL of the original plastid preparation). After 30 minutes of colour development absorbances were measured at 540 nm using a Beckman model DU-40 spectrophotometer and the concentrations calculated using a Quant-II Linear soft-pac module.

***In vitro* Fatty Acid Synthesis**

Fatty acid synthesis was assayed as described by Kleppinger-Sparace *et al.* (1992). The standard reaction mixture contained the following cofactors: 0.5 mM NADH, 0.5 mM NADPH, 0.5 mM CoA, 1 mM $MnCl_2$, 1 mM G3P, 15 mM $KHCO_3$, 6 mM $MgCl_2$, 6 mM ATP, and 160 μM cold acetate + 65 μM ^{14}C-acetate (to a total of 16 μCi/μmole). Stock solutions of the various cofactors were kept at -20°C, 2-500 μL ampules of ATP and NADPH stock solutions ensured minimal degradation due to freezing, thawing, and excess handling. Any specific co-factor additions or deletions per experiment were accommodated by the addition of water to each reaction mixture. The final reaction volume was 500 μL per assay. Normally, a larger batch of standard reaction mix containing the above cofactors sufficient for the number of required

experimental replicates, plus one, was made prior to plastid isolation, thereby facilitating rapid use of the prepared plastids. Differences in treatments per tube were made ahead of the mix while the addition of Tween (if any) was added to the mix directly. A total of 260 μL of reaction mixture was thus dispensed per tube coupled with 200 μL of incubation buffer (0.25M Bis-Tris-propane buffer, pH 7.9, and 0.625 M sorbitol per 0.5 mL total reaction volume). The reaction was initiated by the addition of 40 μL of the purified plastids, which corresponded to an average of 80 μg protein. Unless otherwise specified, incubations lasted for 1 hour in a 25°C water bath. The reactions were gently shaken intermittently at least twice during the incubation period.

Lipid Extraction

Reactions were halted by the addition of 3 mLs of chloroform:methanol:acetic acid (50:100:5 v/v) and vortexed. One mL of 1M KCl and 1 mL of chloroform were then added and the samples vortexed followed by centrifugation for 1 minute at top speed in a benchtop centrifuge. The upper aqueous methanol layer (containing the water soluble fatty acid derivatives and unreacted precursor) was removed via aspiration and the chloroform layer washed with 1.5 mLs of water:chloroform:methanol (3:48:47 v/v) via vortexing and centrifugation for 1 minute. After removal of the second

aqueous phase as before, the final lower chloroform layer containing the radioactive lipid products was removed, capped under N_2 and placed in the -20°C freezer.

Product Analysis

The total radioactivity incorporated was determined by drying the samples under N_2, resuspending them in 1mL chloroform and taking 2 replicates of 25 or 50 μL aliquots for scintillation counting. The counts achieved were then used to calculate the total radioactivity incorporated. Sample protein content was used to calculate the specific activity of each reaction.

Thin layer chromatography was used to separate the radioactive products of lipid metabolism. Plastic backed Brinkman polygram SIL G/UV-254 silica gel TLC plates were pre-run with acetone:acetic acid:water solvent (100:2:1 v/v) and allowed to dry in the hood overnight. The sample and standard application sites were marked in pencil directly on the plates prior to application. The size of the application band varied with the number to be added per plate, but was generally 1.5 cm long, 2 cm from the bottom edge and 1.5 cm apart. Known lipid standards were placed between the samples.

After the samples were dried under flowing N_2, 35 μL of chloroform was washed down the sides of the tube and the sample was then applied to the plate with a drawn-out pasteur pipette. An additional 35 μL of chloroform was used in the same manner to remove any residual sample. The standards were applied at a rate of 30 μg/lipid.

After all the samples and standards were applied, TLC plates (generally two plates per tank) were placed in a glass TLC tank containing acetone:acetic acid:water (100:2:1) and two filter paper saturation pads. The TLC plates were then developed until the solvent front was 1 cm from the top edge. The plates were then removed and placed in a tank under flowing N_2 for two hours. Once dry they were re-developed in another tank containing the second solvent mix consisting of chloroform:methanol:ammonium hydroxide:water (65:35:2:2) which was allowed to ascend only halfway up the plate. The plates were then dried under flowing N_2 for 1 hour. This double solvent system resolved neutral lipids in the upper half of the plate and polar lipids in the lower half of the plate.

Once the plates were dry they were taped in X-ray film holders and Kodak X-Omat AR film was exposed to them in complete darkness for 10 days. The film was then developed and the standard lanes cut from the plates in order to

visualize the lipids upon exposure to iodine. Then with the help of the standards and the developed film the lipids on the plates were outlined in pencil, photocopied, and scraped with a razor blade into either scintillation tubes for radioactivity measurements or glass centrifuge tubes for methyl-ester production.

Preparation of fatty acid methyl-esters was preformed in essentially the same manner as described by Browse *et al.* (1986). Two mLs of 3N methanolic-HCl was added to the samples followed by 1 mL of methanol, the samples flushed with N_2 and placed in a 85°C water bath and shaken periodically for 60 minutes. The tubes were then cooled on ice, 0.5 mL of 0.9%NaCl added and the tubes vortexed. The samples were then extracted three times with 3 mLs of hexane. After vortexing the tubes were centrifuged for one minute and the upper hexane phase containing fatty acid methyl esters was removed via pasteur pipette and transferred to a clean tube. The samples were stored under N_2 in the freezer. Twenty-five microliter aliquots were then removed after the samples had been dried under N_2 and resuspended in 1 mL hexane. Depending on the amount of radioactivity recovered a suitable volume of hexane was then added to the dried sample for subsequent analysis by radio-gas chromatography.

The samples were injected directly into a varian model 340 gas chromatograph (GC) attached to a Packard model 894 gas proportional counter. The GC contained a 1.8m x 4mm glass column packed with 10% CSP-509 on Chromosorb W, and a stream splitter which allowed 80% of the effluent to enter the radioactivity counter. The column was operated isothermally at 185°C while the injector and detector were at 275°C and 300°C, respectively. A Varian Star GC computer program was used to quantify the radioactivity and mass data. Samples were run for 15 minutes and the fatty acid peaks identified by co-chromatography with known standards.

Positional Analysis

In order to improve ^{14}C incorporation the specific radioactivity was increased to 39 μCi/μmole, the incubation time to 2 hours and numerous samples combined (see Table 3 legend). Radiolabelled glycerolipids were separated and recovered from TLC plates via 3 elutions with chloroform:methanol (1:1) and 3 elutions with methanol. The samples were then dried under flowing N_2 and redissolved in chloroform before aliquots were taken for radioactivity measurement and predigest analysis. Each sample was spiked with 300 μg of the corresponding lipid standard and 75 μL of Triton (from a stock of 30 μg/mL chloroform) before being dried down under nitrogen. Sonication was preceded by the addition of 500 μL of Tris (pH7.5, 100 μM) for those

prepared for *Rhizopus* digestion while those to be digested by the phospholipase received the same Tris containing 10 mM $CaCl_2$. Phospholipase A_2 (50 units) from cobra venom was used to digest the *sn*-2 position of PA, PC, SQDG, PG and DGDG while *Rhizopus arrhizus* lipase (500 units) was used to digest the *sn*-1 of MGDG, DG and TG (Sparace and Mudd, 1982; Bishop *et al.*, 1985). The digestions were initiated by the addition of the appropriate enzyme and shaken at 37°C for 3 hours. Digestions were halted by immersing the treatment tube in boiling water for 5 minutes followed, after cooling, by the addition of chloroform:methanol:acetic acid (50:100:5) and extraction as described earlier. The digestion products were then separated by TLC. Plates containing the neutral lipid digests were developed twice with hexane:ether:acetic acid (80:20:1) while plates containing the phospholipids and galactolipids were developed with acetone:acetic acid:water (100:2:1) followed by chloroform:acetone:methanol:acetic acid:water (50:20:10:15:5).

Ten large scale total lipid extractions of intact plastids (100-500 μg lipid each) were prepared for endogenous fatty acid and glycerolipid analysis as described earlier. These analyses were performed by Dr. M. Keith Pomeroy of Agriculture Canada (Ottawa).

Experimental Design

All treatments within each experiment were performed in duplicate, and all experiments were performed at least twice. Data points in the figures are the average of the two replicates.

RESULTS AND DISCUSSION

Endogenous Lipid and Fatty Acid Compositions of Pea Root Plastids

In order to adequately assess galactolipid biosynthesis in relation to the synthesis of fatty acids and other glycerolipids as described in this study, it is important to have an accurate estimate of the endogenous lipids and fatty acids comprising pea root plastids. The endogenous lipids and fatty acids have been previously examined in pea root plastids (Xue, 1993). However, those results were based one single experimental analysis and thus required verification. Table 1 summarizes the current results of this verification study. In this study, MGDG is the most prevalent lipid, representing 15 mol%. This is followed by TAG at 13% and DGDG, DAG, FFA and PC all at ~10%. SQDG, PA and PG were found in the smallest amounts at 1, 2 and 4% respectively. This current work reports a lower % of PA, PC and PG (1/4, 1/2 and 1/6 respectively), while DGDG, MGDG, FFA and DAG all

Table 1. Fatty Acid Analyses of Endogenous Pea Root Plastid Lipids (mass %) (averages of 2 replicates representing a total of 5 pooled plastid samples)

Lipid	%*	14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
PA	2	1	14	4	2	1	8	26	37	3	1	2	1	1
PC	11	-	13	-	-	-	4	7	61	13	-	1	-	-
SQDG	1	3	17	8	8	3	9	14	22	8	4	5	-	-
PG	4	1	39	3	1	1	6	9	26	13	-	1	-	-
DGDG	10	-	5	-	-	-	4	5	21	64	-	-	-	-
MGDG	15	-	2	-	-	-	1	3	24	68	-	-	-	-
FFA	11	3	15	4	1	1	10	23	30	2	2	2	3	4
DAG	10	2	15	1	-	-	6	33	24	5	2	3	3	6
TAG	13	1	7	1	-	-	4	35	35	8	1	3	1	4

* represents the lipid mole % average, the other 23% are in SE(7%),MAG(5%),ASG(5%),LPA(3%), LPC(1%) and PI, PS (both <1%).
- represents less than 0.5%

had percentages over 2 times that of the previous data. These differences are likely due to prolonged storage of Xue's lipid samples under less than ideal conditions which may have resulted in some lipid degradation.

16:0, 18:0, 18:1, 18:2, and 18:3 generally represented 75 to 100% of the fatty acids in all lipids. Small amounts of C_{16} unsaturated fatty acids and very long C_{20} and C_{22} fatty acids were also detected especially in PA, SQDG and the neutral lipids. PG had the highest amount of 16:0 at 39%, followed by SQDG, FFA, DAG, PA and PC all at 14-17% while MGDG, DGDG and TAG had the least 16:0 at 2-7%. Correspondingly MGDG, DGDG, TAG and PC all had over 80% of their fatty acids as C_{18} moieties. These were followed by PA, DAG and FFA at ~70% and SQDG and PG at ~50% C_{18}. These observations suggest that pea root plastids, like pea chloroplasts, may greatly rely on the eukaryotic pathway for assembly of its membrane glycerolipids. However, the precise relative contributions of the eukaryotic vs. prokaryotic pathways for assembly of pea root plastid glycerolipids cannot be determined in this brief study alone.

In terms of fatty acid unsaturation, most of the 16 carbon lengths were saturated while the 18 carbons were not. MGDG and DGDG had the most 18:3 at 68 and 64% respectively. PC had the highest 18:2 at 61% while the other lipids had 18:2

in the 20-30% range. Oleate was present in this same range for PA, FFA, DAG, and TG. Relatively little saturated 18 carbon fatty acid (18:0) was found. The highest was 10% in the FFA fraction. This suggests the availability of O_2 and reduced nucleotides were adequate for 18:1 production before plastid isolation. Combined with the low amounts of 16:3, this is consistent with the idea that pea is an "18:3" plant (Joyard *et al.*, 1993; Heemskerk *et al.*, 1990) in regard to both the root plastids and the leaf chloroplasts.

In comparison to roots the MGDG of pea leaf chloroplasts (Heemskerk *et al.*, 1990) has 91% 18:3. The plastids isolated here have 68% of endogenous MGDG as 18:3 and 24% as 18:2. This difference may be attributed to the level of oxygen available for desaturation or that such polyunsaturation is not a physiological characteristic of pea root plastids. Turnip root has been reported to have 12% 16:0 and 56% 18:3 in its MGDG (Lepage 1967)- this level is close to the pea root plastid's 68% in 18:3, but not its 2% 16:0. The total lipid composition of soybean roots have an almost equal 3-way split between 16:0, 18:2 and 18:3 (Terzaghi, 1989). The results with turnip and soybean however are for the whole root, not just the plastids within them. Potato amyloplast envelope lipids (Fishwick and Wright, 1980) have a fatty acid distribution similar to that of pea root plastids in regard to PC and PG. However a

lower percentage of 18:3, and a higher percentage of 18:2, were found in the potato's MGDG and DGDG. The DAG within the pea root plastids presented here have higher proportions of 16:0 and 18:1 but a lower amount of 18:2 than potato. These differences are likely due to the different origins and regulation of lipid synthesis in these various plastids.

Effects of UDP-Galactose Concentration and Incubation Time

Galactolipid biosynthesis in these plastids was completely dependent on an exogenous supply of UDP-galactose. This was expected since UDP-galactose, as well as the enzymes involved in UDP-galactose synthesis, are found in the cytosol (Konigs and Heinz, 1974). MGDG synthase, however, is a plastid envelope enzyme (Cline and Keegstra, 1983; Joyard *et al.*, 1993). UDP-galactose had two effects on glycerolipid synthesis from acetate. First UDP-galactose caused an increase in the total incorporation of ^{14}C-acetate into the glycerolipids up to a concentration of 75 μM (figure 3). At this concentration, acetate incorporation into lipid was stimulated approximately 60%. This suggests that UDP-galactose may have a regulatory effect on one or more of the enzymes involved in fatty acid and ultimately glycerolipid production. In this regard Qi and Sparace (1994) have shown that UDP-galactose can increase the activity of ACCase by approximately 40%. Second, UDP-galactose caused a marked increase in the proportion of MGDG

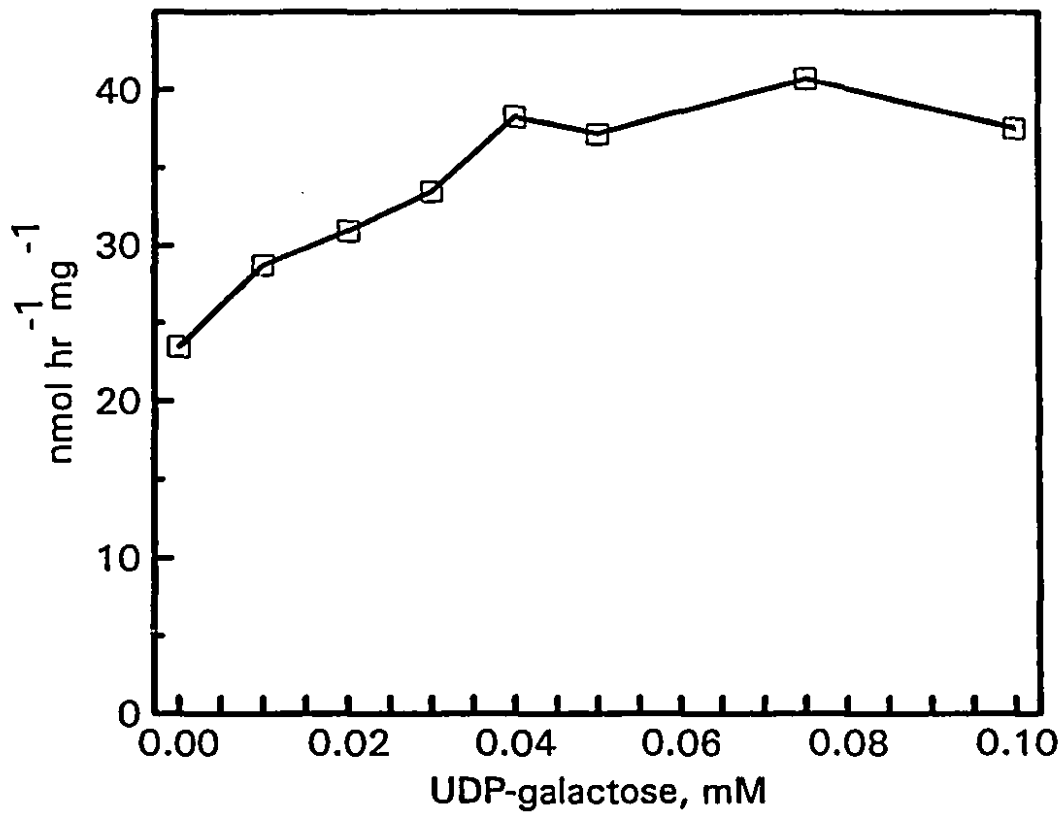


Figure 3. The effect of UDP-galactose concentration on total ^{14}C-acetate incorporation in pea root plastids. Incubations were for one hour as described in "Materials and Methods".

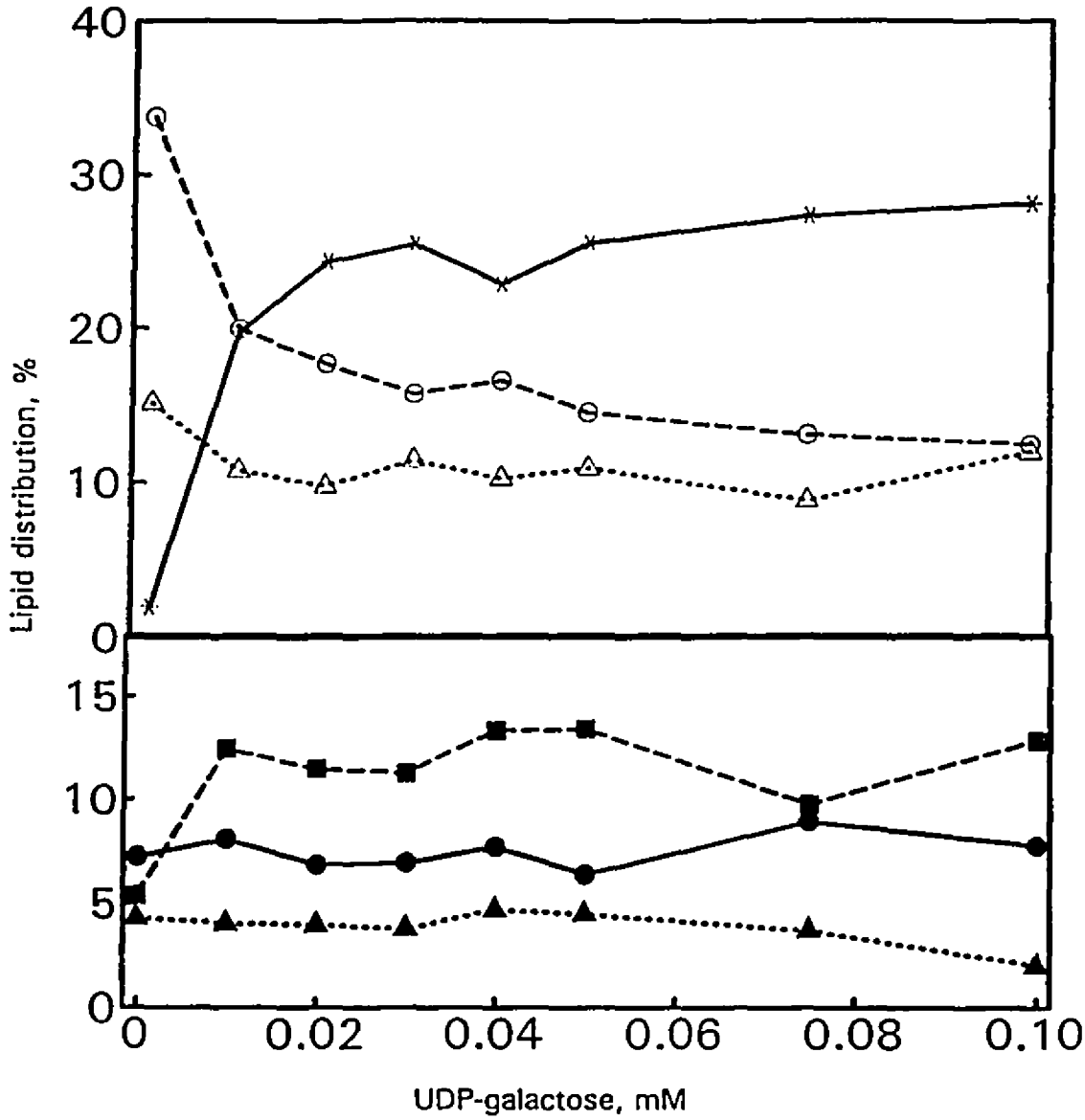


Figure 4. The effect of UDP-galactose concentration on percent distribution of ^{14}C-acetate labelled lipids in pea root plastids. The remaining labelled lipids were fairly constant: PG (9%), MAG (5%), L-PC (2%), SQDG (2%) an the label present at the solvent front and origin average 6% and 3% respectively. Incubations were for one hour as described in "Materials and Methods".

✳ MGDG, O DAG, △ PA, ● PC, ■ FFA, ▲ TAG

synthesized (figure 4). Up to 30% of the total radioactivity was recovered in MGDG in one hour incubations. This effect on MGDG biosynthesis was essentially saturated at 20 μM UDP-galactose. At the same time there was a corresponding 20% decrease in the amount of diacylglycerol accumulated (figure 4). This is expected since DAG is a co-substrate for MGDG biosynthesis. UDP-galactose had little or no effect on the amounts of other glycerolipids synthesized, although a slight increase in FFA and a slight decrease in PA was seen upon the initial addition. It is interesting to note that DGDG was not detected. In most of the experiments presented here, DGDG synthesis represented 0 to 2% of the total radioactive lipids detected in a one hour incubation (<10% of the incorporation into MGDG).

UDP-galactose dependent galactolipid biosynthesis from ^{14}C-acetate in pea root plastids is markedly greater than that shown for plastids of other 18:3 plants or chloroplasts from the same plant. Little or no biosynthesis is observed with pea chloroplasts (Joyard and Douce, 1987), daffodil chromoplasts (Liedvogel and Kleinig, 1979), or sycamore amyloplasts (Alban *et al.*, 1989). As mentioned earlier, plastids from 18:3 plants are thought to have little or no PA phosphatase to generate a sufficient amount of the DAG required for galactolipid synthesis. These observations suggest that the presence or regulation of PA phosphatase in

pea root plastids is markedly different from that of other 18:3 plastids and may be more similar to that of 16:3 plastids. The effective concentration of UDP-galactose in this investigation, however, is comparable to or slightly less than that of other workers. Heemskerk *et al.* (1987) using spinach chloroplast envelopes, found UDP-galactose:1,2-diacylglycerol galactosyltransferase activity saturated after 0.1mM UDP-galactose. Coves (1988), also found a K_m value of 100μM UDP-galactose for MGDG synthase in spinach chloroplast envelopes. It is interesting to note that in spinach UDP-galactose is reported to be in the amount of 0.5mM in the cytosol (Joyard *et al.*, 1993).

At an intermediate concentration of UDP-galactose (75μM), total *14*C-acetate incorporation was linear for approximately 2 hours but continued to increase for up to six hours (figure 5). Over the same time period, MGDG synthesis was linear for approximately 1 hour (figure 6). The remaining lipids showed changes consistent with established product-precursor relationships for acetate-labelled lipids. At the shortest time (5 minutes) radioactivity was greatest in FFA which gradually decreased. PA followed by DAG both rapidly accumulated label (37% at 5 minutes and 26% at 15 minutes, respectively). Both rapidly declined as MGDG accumulated. Similar results were obtained when pea root plastids were first preincubated with acetate for one hour prior to the

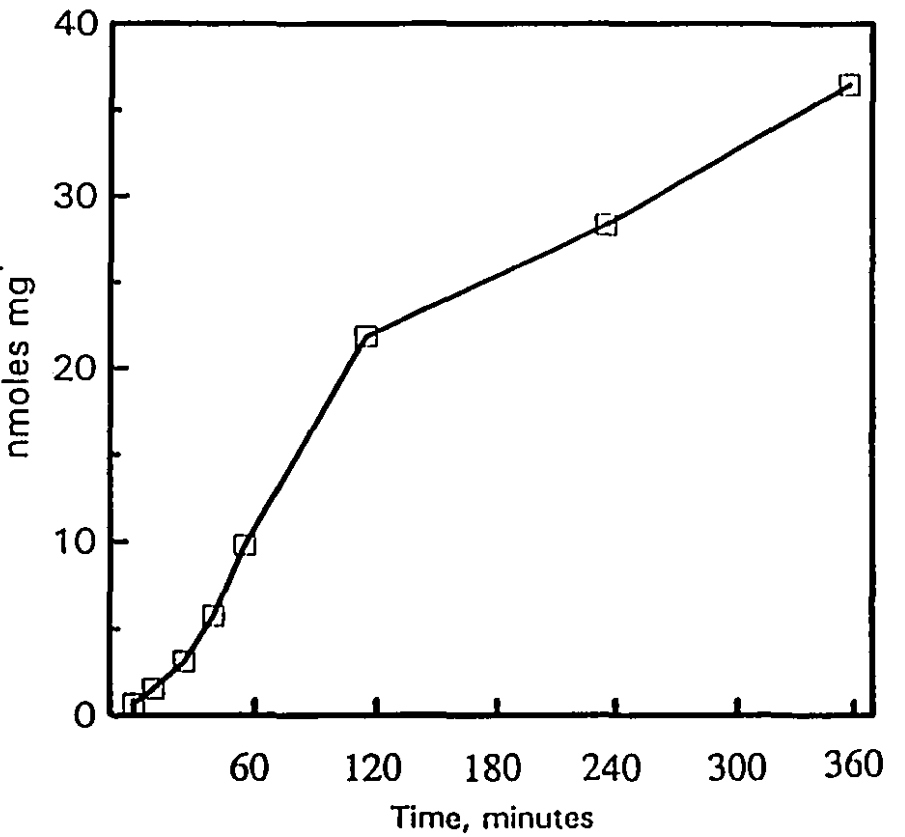


Figure 5. The effect of incubation time on total ^{14}C-acetate incorporation in pea root plastids. Incubations contained 0.075mM UDP-galactose.

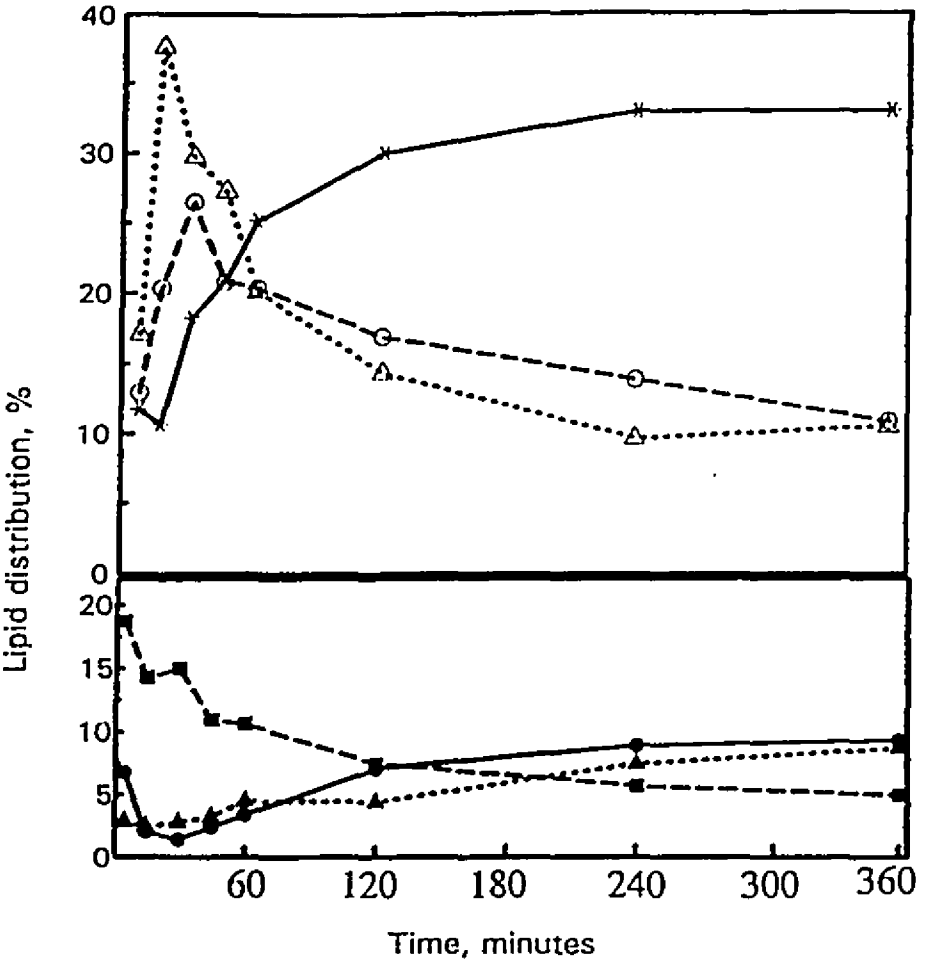


Figure 6. The effect of incubation time on percent distribution of ^{14}C-acetate labelled lipids in pea root plastids. The remaining labelled lipids were fairly constant: SQDG averaged 7.5% (5-11), DGDG (3%), PG (5%) and the label present at the solvent front and origin averaged 4% and 3% respectively. Incubations included 0.075mM UDP-galactose.

✳MGDG, ○ DAG, △ PA, ● PC, ▬ FFA, ▲ TAG

addition of UDP-galactose (figures 7 and 8). Similarly when UDP-^{14}C-galactose was used instead of ^{14}C-acetate, figure 9 also illustrates a rapid incorporation of UDP-^{14}C-galactose within the first hour of incubation. With either precursor, labelled DGDG was not routinely detected.

In a similar experiment with cauliflower bud plastids, Journet and Douce (1985) followed the incorporation of ^{14}C-G3P into lipid. They found a similar decrease in DAG upon the addition of UDP-galactose and MGDG synthesis. Miquel and Dubacq (1992), using ^{14}C-acetate in pea chloroplast envelope membranes, found a decrease in PA while DAG and MGDG percentages increased. The proportion of radioactivity in MGDG was much lower (<10 %) than that presented here. This is most likely due to the transfer of MGDG into the thylakoids (Morre *et al.*, 1991; Rawyler *et al.*, 1992) since they incubated the chloroplast plastids followed by isolation of the membranes for analysis.

The data shown in figures 3 and 8 (prior to the addition of UDP-galactose) are in good agreement with the previous studies of glycerolipid biosynthesis in pea root plastids (Xue, 1993). The rate of acetate incorporation into lipid obtained here (23 nmoles/hr/mg) was similar to the 22 nmoles/hr/mg reported by Xue (1993). The distribution of radioactivity among lipids was also similar to that of Xue

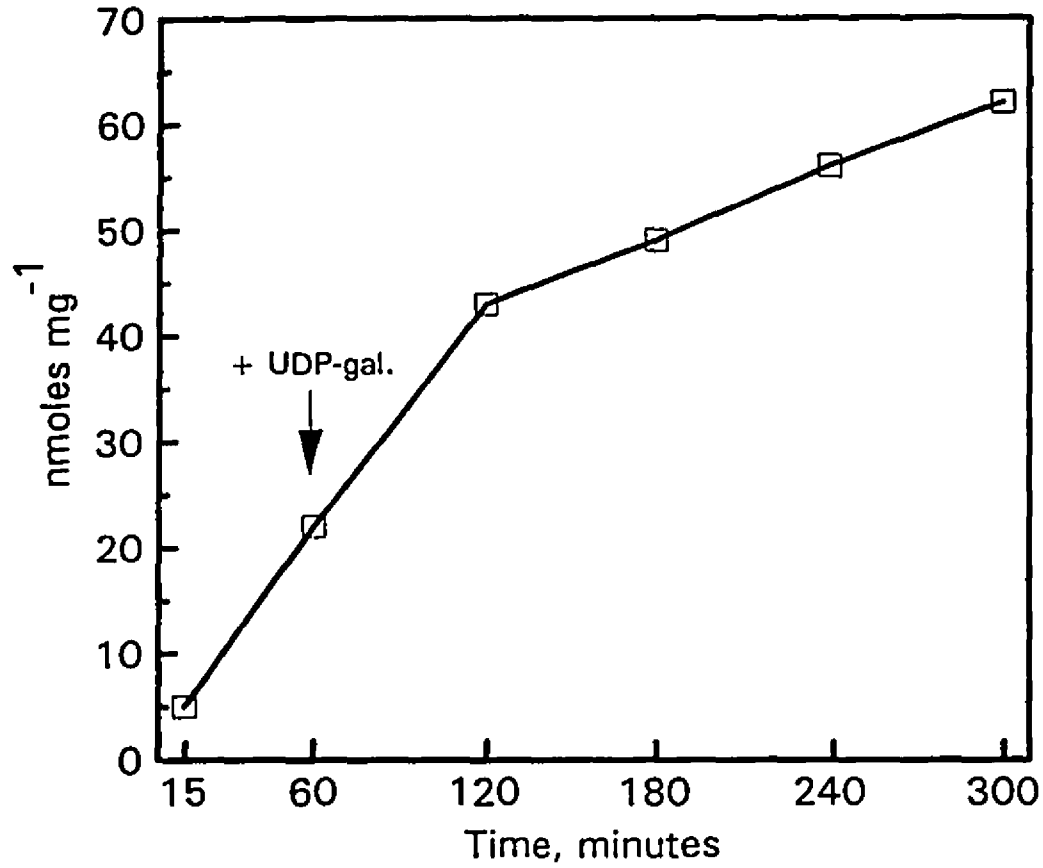


Figure 7. The effect of incubation time on total ^{14}C-acetate incorporation in pea root plastids after the addition of UDP-galactose at 60 minutes. The addition was 0.075mM UDP-galactose.

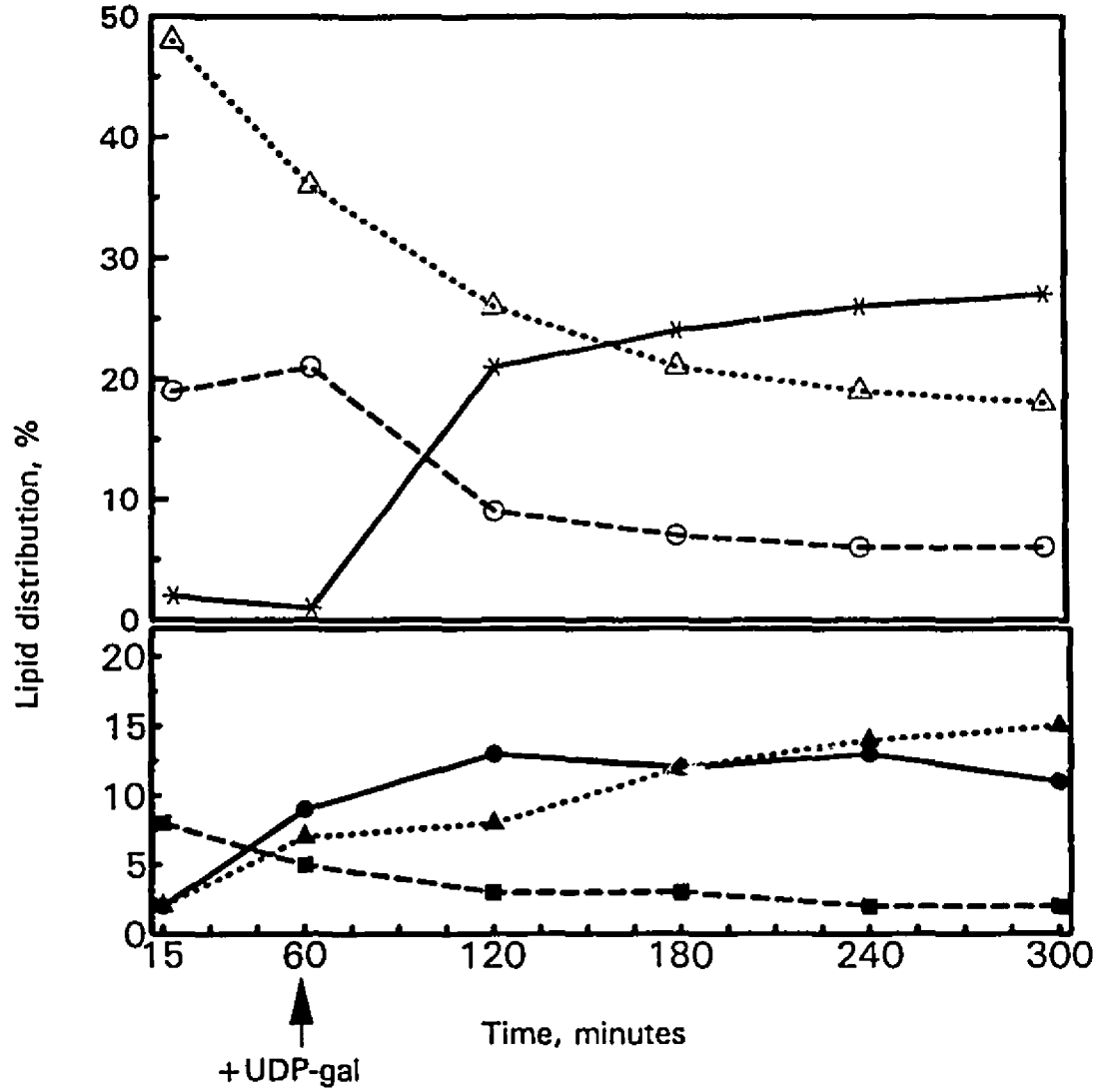


Figure 8. The effect of incubation time on percent distribution of ^{14}C-acetate labelled lipids in pea root plastids after the addition of UDP-galactose at 60 minutes. The addition was 0.075mM UDP-galactose. The remaining labelled lipids were PG (7%) and the label present at the origin (4%).

✳ MGDG, ○ DAG, △ PA, ● PC, ■ FFA, ▲ TAG

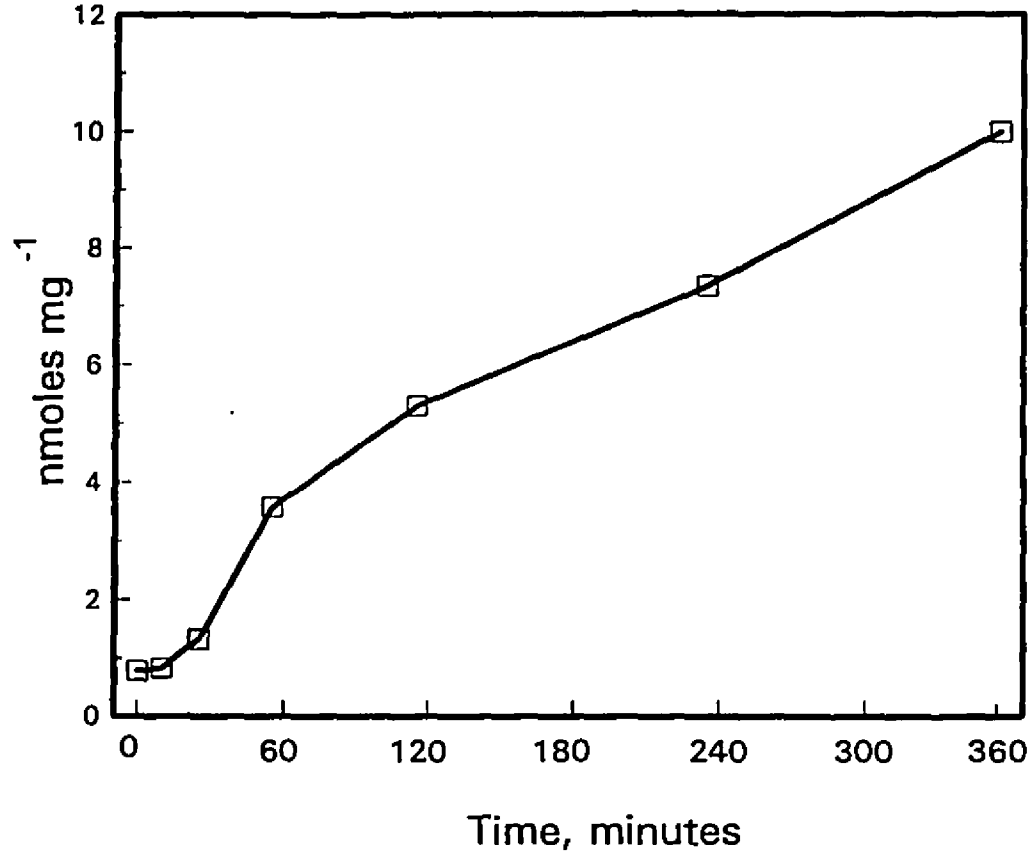


Figure 9. The effect of incubation time on total UDP-^{14}C-galactose incorporation in pea root plastids. Incubations contained 0.2mM acetate and 0.038mM UDP-^{14}C-galactose (4.22μCi/μmole).

(1993) with most radioactivity (40%) recovered in PA followed by DAG (18%), and lesser amounts in PG, PC, and TAG.

In terms of percent of labelled acetate incorporated into MGDG, the results obtained here are comparable to that found in photosynthetic tissues and chloroplasts (Sparace and Mudd, 1982; Heemskerk *et al.*, 1988; Mudd *et al.*, 1985; Bertrams *et al.*, 1981). These results indicate that the ability to synthesize MGDG is still present after root differentiation even though these are nonphotosynthetic, nongreening plastids. Further, in contrast to other 18:3 plastids, pea root plastids appear to contain sufficient PA phosphatase activity to produce adequate amounts of DAG necessary for high rates of galactolipid biosynthesis.

In comparison to the endogenous lipid composition of pea root plastids (Table 1), after one hour incubation with 75μM UDP-galactose, the labelled MGDG represents almost 2x that of the endogenous MGDG and essentially no DGDG (down from 10%). The lack of DGDG could be due to different enzymatic requirements for DGDG synthesis (Heemskerk *et al.*, 1987). The enzyme is known to prefer eukaryotic 18:3/18:3-MGDG as a substrate (Roughan, 1987) and is located on the cytosolic side of the outer envelope membrane (Van Besouw and Wintermans, 1978; Dorne *et al.*, 1982; Alban *et al.*, 1988).

It follows that this prokaryotic incubation system would produce little DGDG. FFA and TAG have a lower representation (1/5 and 1/2 respectively) in the radiolabelled lipids than the endogenous while PA is over 10x higher after a one hour incubation. This suggests that TAG (as a competitor with MGDG for DAG and FFA) is produced less as PA and total incorporation of acetate is stimulated upon MGDG synthesis.

Radioactive Fatty Acid Analysis of Acetate-labelled Lipids

The radioactive fatty acid composition of ^{14}C-acetate labelled lipids synthesized by pea root plastids is summarized in Table 2. In order to improve ^{14}C incorporation the specific radioactivity was increased to 39μCi/μmole, the incubation time to 2 hours and numerous samples combined (see Table 2 legend). Palmitate is the predominately labelled fatty acid in all lipids (50-80%) except in PC where 18:1 represents 80% of the radioactive fatty acids. MGDG and DGDG have the most 16:0 at 80% followed by DAG, PA and PG at ~70% and SQDG and TAG at ~50%. Forty percent of the fatty acids found in FFA are 16:0 resulting in an almost 3-way split between the fatty acids. Most of the lipids have an almost equal amount of 18:0 and 18:1 (10-20%). The exceptions are PC with 80% 18:1 and 4% 18:0 and DAG with 16% 18:0 and 9% 18:1.

Table 2. Radioactive fatty acid compositions of ^{14}C-acetate labelled lipids in pea root plastids. Detection was enhanced by combining the samples (percentages represent an average of 4 replicates, each containing 3 samples), increasing the incubation time to 2 hours, and increasing the specific activity of the label. Incubations contained 0.268 mM ^{14}C-acetate (39μCi/μmole) and 0.075 mM UDP-galactose.

Lipid	%*	16:0	18:0	18:1
PA	23	72	13	15
PC	5	16	4	80
SQDG	3	59	17	24
PG	6	69	17	14
DGDG	7	81	11	8
MGDG	26	80	10	10
FFA	11	40	29	31
DAG	10	75	16	9
TAG	4	53	24	23

* represents the average % ^{14}C in each lipid. The remaining 5% was in the residues and lyso-PA at the origin of the TLC plates.

In comparison to the endogenous fatty acid compositions of the various lipids, the labelled fatty acid compositions generally show markedly higher amounts of 16:0 (with the exception of PC) with corresponding higher amounts of C_{18} fatty acids. In PA 16:0 increases from 14 to 72%, SQDG 17 to 59%, PG 39 to 69%, DGDG 5 to 81%, MGDG 2 to 80%, FFA 15 to 40%, DAG 15 to 75% and TAG 7 to 53% 16:0. These observations suggest that the elongation of 16:0 to stearate and its subsequent desaturation to oleate may be somewhat restricted under the *in vitro* conditions used here. More importantly, isolated plastids would necessarily rely exclusively on the prokaryotic pathway for assembly of ^{14}C-labelled lipids since the normal source of C_{18}-fatty acid enriched DAG moieties (the cytosol) is no longer present. As mentioned earlier, the prokaryotic, (or plastid) pathway favours the utilization of C_{16} fatty acids while the eukaryotic (or cytosolic) pathway favours the utilization of C_{18} fatty acids. The relatively high amounts of 18:0 (an average of 16% in radioactive lipids vs. 6% endogenous) may reflect limiting oxygen levels in *in vitro* incubations. Similar proportions of radioactive 16 and 18 carbon fatty acids were reported in earlier experiments on glycerolipids and fatty acids in pea root plastids (Xue, 1993; Stahl and Sparace, 1991). The primary exceptions were with DAG and FFA. The current work has a higher percentage of 16:0 in DAG (75% vs. 52%) and a lower percentage of 16:0 in the FFA

(40% vs 79%) as compared to previous studies. The current work also has a higher amount of 18:0 in the various lipids (10-29% vs. 0-13%). The main difference between these previous studies and those represented here is the addition of UDP-galactose in this study. These differences suggest that UDP-galactose results in increased use of 16:0 in DAG with the production of MGDG.

Envelopes from the chloroplasts of pea show an endogenous level of 19% 16:0, 20% 18:2 and 53% 18:3 (Miquel and Dubacq, 1992). When incubated with ^{14}C-acetate for one hour the outer envelope contained 8% 16:0 and 92% 18:1 while the inner envelope had 19% 16:0 and 81% 18:1. The endogenous levels of the total lipids presented here indicate on the average 14% 16:0, 31% 18:2 and 20% as 18:3 as well as considerable levels in less unsaturated species of 18 carbon fatty acids. Upon incubation, the radioactive fatty acid totals represent on the average 60% 16:0, 16% 18:0, and 24% 18:1. These are significant differences with Miquel and Dubacq considering the pea root plastid is devoid of thylakoids and the chief membrane being left is therefore the envelope. However, these results are not directly comparable considering the chloroplast envelopes were extracted after incubation therefore a possible selective transfer to the thylakoids may have resulted (Morre *et al.*, 1991; Rawyler *et al*, 1992). The chloroplasts also likely

have different pools of cold precursors since they were likely producing (as well as transferring) galactolipids prior to isolation and incubation.

Positional Analysis of Radioactive Fatty Acids in Glycerolipids

Table 3 summarizes the distribution of radioactive fatty acids with respect to their positions on each lipid. Most of the label (i.e. those fatty acids produced during the incubation with ^{14}C-acetate) is associated with the *sn-2* position of PA and PC (>90%) and to a lesser extent SQDG, PG, DGDG and MGDG (all ~70%). The neutral lipids, DAG and TAG, however, had the greatest percentage of label associated with the *sn-1* (plus *sn-3*) positions. One explanation for the unequal distribution of fatty acids in the polar lipids could be that these lipids are synthesized from a pre-existing pool of lyso-PA. As this pool is depleted during *in vitro* incubations (as when galactolipid synthesis is favoured, see figure 4), a greater amount of label would occur in the *sn-1* position. Alternatively, some acyl exchange may occur between a *de novo* synthesized fatty acid pool and previously formed glycerolipids.

The distribution of acyl radioactivity in DAG and TAG is more difficult to explain. Clearly the ^{14}C-acyl distribution in ^{14}C-DAG does not reflect that which might be derived from

Table 3. Distribution of radioactivity among positions of digested lipids labelled with ^{14}C-acetate in pea root plastids. Detection was enhanced by combining the samples (data represents an average of 3 replicates, each containing 19 samples). One replicate was also incubated for 2 hours with an increased specific activity of the label (0.27mM ^{14}C-acetate from 39 increased to 49μCi/μmole). Incubations contained 0.075mM UDP-galactose.

Lipids		total % of digest	14:0	16:0	16:1	18:0	18:1
PA	*sn2*	94	-	80	-	3	17
	sn1	6	-	28	-	25	47
PC	*sn2*	97	-	7	3	-	90
	sn1	3	-	22	-	9	68
SQDG▲	*sn2*	74	-	49	-	-	51
	sn1	26	-	57	-	4	39
PG	*sn2*	77	-	82	6	5	7
	sn1	23	-	35	-	26	39
DGDG▲	*sn2*	77	-	79	-	6	15
	sn1	23	-	74	-	22	5
MGDG	*sn2*	68	-	96	-	4	-
	sn1	32	-	32	-	24	44
DAG*	*sn2*	19	9	76	-	6	9
	sn1	73	6	36	-	22	36
TAG**	*sn2*	29	9	63	-	9	19
	sn1,3	42	-	62	-	14	24

- denotes not detected
▲ due to low counts these only include one rep. of high counts
* the remaining 8% of digest went into production of TAG thereby decreasing the % in *sn1*
** the remaining 29% of digest was in DAG which includes positions *sn1* and *sn2*

PA for membrane glycerolipid biosynthesis. It is possible that ^{14}C-DAG and TAG represent a mechanism of storage lipid assembly that is somehow segregated from membrane lipid assembly. Further, PA's and DAG's targeted for storage lipid biosynthesis may be subjected to acyl modification preferentially at position 1 as in some oilseeds (Browse and Somerville, 1991). This idea is supported by the data of table 1 showing C_{20} and C_{22} fatty acids primarily in PA, DAG, and TAG. However, under the *in vitro* conditions used here, these very long chain fatty acids are not available, and thus the *sn-1* fatty acids are replaced with *de novo* synthesized fatty acids. Alternatively, the low percentage of radioactive fatty acids in *sn-2* could indicate a selective use of a cold pool of fatty acid for this position or perhaps acyl exchange between lipids. It is interesting to note that DAG is also the only lipid where 14:0 was found in both positions. The radioactive fatty acid composition at the *sn-1* position of MGDG is similar to that of PA, PG and DAG which is consistent with the known pathway of prokaryotic synthesis.

In terms of the radioactive fatty acids found at each position, the *sn-2* position of most lipids contains predominantly (~80%) 16:0. This is to be expected since undigested radioactive lipids contained predominantly 16:0 (table 2) which was subsequently shown to be located

primarily on position *sn-2*. The *sn-1* position is more variable in it's fatty acid composition, but notably generally contains greater amounts of C_{18} fatty acids than C_{16} fatty acids. These patterns largely reflect the specificities of the plastidic acyl transferases of the prokaryotic pathway for lipid assembly. However, PC and SQDG stand out in terms of their radioactive fatty acid compositions. PC has 90% 18:1 at position *sn-2*, illustrating that the synthesis of this lipid in plastids remains poorly understood. Earlier work with chloroplasts suggested that PC was synthesized via the acylation of lyso-PC (for a review see Browse and Somerville, 1991). The data of table 3 for PC are consistent with this hypothesis. SQDG is somewhat unique in that it contains essentially equal amounts of 16:0 and 18:1 at position *sn-2*, despite the known specificity of the second acyl transferase. These observations suggest that some selectivity may exist in isolated pea root plastid lipid metabolism such that DAG molecular species enriched in C_{18} fatty acids at *sn-2* are routed towards SQDG synthesis while DAG molecular species enriched in C_{16} fatty acids at position *sn-2* are routed towards galactolipid synthesis.

Previous work done on pea root plastids without UDP-galactose showed a similar pattern of unequal labelling of positions *sn-1* and *sn-2* of various glycerolipids (Xue,

1993). The phospholipids (other than PA) in those experiments also had the highest amount of radioactivity in *sn-2* (63-70%), while the neutral lipids had higher proportions in *sn-1* (77-79%). In contrast to the results shown here, the PA in Xue's experiments had essentially equal radioactivity in the two positions, while the work presented here had 94% in *sn-2*. The PC presented here is also higher at 97% in the *sn-2*.

Effects of Cofactor Deletion

Table 4a and 4b illustrate the effect of cofactor deletion on total and individual glycerolipid biosynthesis from labelled acetate using two different concentrations of UDP-galactose. In general, the greatest effects were observed on the rate of total glycerolipid biosynthesis with relatively small effects on the amounts of MGDG synthesized. These results largely reflect the cofactor requirements for fatty acid biosynthesis (since acetate is the precursor) and are in agreement with previous studies of fatty acid and glycerolipid biosynthesis in these plastids (Stahl, 1990; Xue, 1993). However, their studies did not include UDP-galactose in their reaction mixtures to emphasize galactolipid biosynthesis.

The greatest effect on total incorporation was observed with the deletion of ATP, and to a lesser extent, CoA. The

Table 4a. The effect of cofactor deletion on ^{14}C-acetate labelled lipids in pea root plastids. Incubations contained 0.075mM UDP-galactose.

	%*	origin	PA	PC	SQDG	PG	MGDG	MAG	FFA	DAG	TAG
control	100	5	36	6	1	7	17	1	12	12	3
-UDP-gal	105	5	37	6	1	8	1	1	13	24	4
-ATP	5	-	-	-	-	-	-	-	-	-	-
$-Mg^{2+}$	87	4	17	7	2	11	18	2	18	15	5
$-KHCO_3$	24	5	22	2	2	3	23	2	17	18	5
$-Mn^{2+}$	105	4	29	6	1	7	21	1	13	13	3
-CoA	18	3	8	2	2	5	21	4	39	14	3
-NADH/NADPH	71	7	30	7	2	11	20	2	9	10	3

* represents total ^{14}C-acetate incorporation as a percent of control
- represents counts too low for reliable measurement

Table 4b. The effect of cofactor deletion on ^{14}C-acetate labelled lipids in pea root plastids. Incubations contained 11μM UDP-galactose.

	%*	origin	PA	PC	SQDG	PG	MGDG	MAG	FFA	DAG	TAG
control 11μM	100	7	35	7	1	4	7	0	13	20	4
control 75μM	108	7	34	5	2	7	15	0	12	14	4
-UDP-gal	101	8	37	4	2	4	1	0	12	26	6
-ATP	3	-	-	-	-	-	-	-	-	-	-
$-Mg^{2+}$	92	5	24	5	2	7	6	0	21	23	7
$-KHCO_3$	18	7	19	3	2	4	7	0	16	33	7
$-Mn^{2+}$	135	6	39	4	2	6	8	0	11	19	5
-CoA	14	3	8	2	3	4	11	0	37	28	6
-NADH/NADPH	72	4	30	8	2	9	11	0	9	20	5

* represents total ^{14}C-acetate incorporation as a percent of control
- represents counts too low for reliable measurement

omission of these cofactors greatly reduced acetate incorporation into lipid by >95% and >82%, respectively. Similarly, the omission of $KHCO_3$ caused an inhibition down to 18-24%. Both ATP and CoA are required for the formation of acetyl-CoA from acetate while ATP and $KHCO_3$ are required for carboxylation of acetyl-CoA to malonyl-CoA (Stumpf, 1984).

The omission of Mg^{2+} reduced total lipid biosynthesis by approximately 10-15%. This was not as pronounced as that shown by earlier workers [25% in studies of Xue (1993)]. Deletion of Mn^{2+} caused an increase in total activity (105-135%) similar to the results obtained for fatty acid and glycerolipid synthesis. Similarly the omission of reduced nucleotides (NADH and NADPH) cause an inhibition similar to Xue's (1993) glycerolipid studies.

The production of MGDG, as a percent of total glycerolipid, in these acetate experiments was most affected by the omission of UDP-galactose from the standard reaction mixture. As expected, this reduced galactolipid synthesis essentially to zero.

In general for the 75μM UDP-galactose experiment (Table 4a), all treatments, particularly those causing reduced total lipid synthesis, resulted in an increase of 3 to 6% in the

proportion of MGDG synthesized. Specifically, the omission of Mg^{2+} had little effect (up by <1%) while that of Mn^{2+} was slightly stimulatory (up by 4%) to the % label in MGDG. Heemskerk *et al.* (1987) found MGDG synthesis did not require cations but was slightly stimulated with the addition of Mg^{2+} and Mn^{2+}. One could speculate that a higher concentration of Mg^{2+} funnels the fatty acids away from PG for use elsewhere (i.e. PA through to galactolipids). There is a light induced release of Mg^{2+} into the stroma of chloroplasts (Joyard *et al.*, 1993), perhaps it therefore is associated with chloroplast thylakoid development and the galactolipids required therein. This effect of Mg^{2+} is similar to that found in spinach chloroplasts (Nakamura and Yamada, 1975). Although high ionic strength is required for assaying galactolipid synthesis (Coves *et al.*, 1988), Mn^{2+} in combination with Mg^{2+} can inhibit activity. Heemskerk *et al.*, (1987) has found, however, that DGDG synthesis is highly stimulated by Mn^{2+} as well as with Ca^{2+} and Mg^{2+}. However, DGDG was not detected here.

The addition of MoO_4^{2-} (Rathmore *et al.*, 1993), or NaCl and $CaCl_2$ (Zhao *et al.*, 1993) has been found to stimulate DGDG synthesis and decrease that of MGDG. Salt stress with NaCl and $CaCl_2$ in wheat seedlings have produced increases in DGDG as well as MGDG in the roots with a concurrent decrease in PA and PG. Menikh and Fragata (1993) found DGDG molecules

aggregate in solutions containing cations as a result of adsorption of the ions in the polar heads. Perhaps the MGDG and/or DGDG's role in thylakoid structure and production are influenced by MGDG phase separation from other lipids by NaCl and $MgCl_2$. The pH could also be a factor here, as Mudd *et al.* (1969) found in spinach chloroplasts that DGDG synthesis was stimulated and MGDG synthesis inhibited if the pH was lowered.

In tables 4a and 4b, the greatest increase in % in MGDG occurred with bicarbonate omission followed by the omissions of CoA and reduced nucleotides, all of which affect fatty acid synthesis (Stahl, 1990). All of these omissions also had reductions in their total incorporation suggesting a favouring toward galactolipid synthesis. At the lesser concentration of 11μM UDP-galactose the largest increases in % MGDG are in CoA and reduced nucleotides. Without these cofactors more of the labelled products from the labelled fatty acids are funnelled toward galactolipid synthesis rather than simply contributing to a glycerolipid pool (such as PA or FFA).

This is confirmed when examining the effects on the other glycerolipids. When MGDG was stimulated with the omission of CoA there was a dramatic decrease in PA (from 36% to 8%) while FFA had a comparable increase. This suggests some

acyl-exchange or a surplus of fatty acids rather than the production of more fatty acids (hence the low incorporation).

With the omission of bicarbonate there was also a decrease in the phospholipids while the % radioactivity in FFA and especially DAG (MGDG precursor) increased. Sanchez and Mudd (1993) have studied bicarbonate effects on acetate labelled lipids in spinach chloroplasts and found an optimum of 5mM bicarbonate for total ^{14}C-acetate incorporation (15mM was used here). In their study increases in DAG and decreases in PG and stearate levels in MGDG were observed. These results are also similar to those for daffodil chromoplasts (Kleinig and Liedvogel, 1978). Externally supplied bicarbonate is used for the production of malonyl-CoA which leads to the formation of PA. This is supported by the lower proportion of PA upon its deletion.

The omission of reduced nucleotides (Tables 4a and 4b) also caused a reduction in PA but a slight increase in PC and PG (1 and 3-5% respectively) coupled with a decrease in FFA by 3-5%. The deletion of the cations Mg^{2+} and Mn^{2+} differed in their effects on the % distribution of glycerolipids. While the omission of both cofactors caused a reduction in PA at 75μM UDP-galactose, that caused by Mg^{2+} omission was greater (down to 17% vs. 29% for Mn^{2+} omission). At 11μM UDP-

galactose there was actually an increase in PA when Mn^{2+} was ommitted. At this lower concentration of UDP-galactose the deletion of Mn^{2+} appears to have an inhibitory action on MGDG synthesis since the total glycerolipid synthesis increased but there was no increase in % MGDG as seen in Table 4a. PA increases and FFA and DAG% decrease in contrast to that at the higher UDP-galactose. It appears that at the lower amount of UDP-galactose, the higher the incorporation rate the more PA is produced. The amount of galactose is the rate limiting factor in this case, with the presence of cations as beneficial to MGDG synthesis.

In similar experiments using UDP-^{14}C-galactose instead of ^{14}C-acetate the omission of cofactors required for fatty acid biosynthesis had little effect on galactolipid biosynthesis (Table 5). The omission of ATP and CoA had the largest effects, both reducing the activity by about 20%. Since these are the two cofactors that most affect fatty acid synthesis it would appear that most of the galactolipids here are derived from endogenous DAG with perhaps 20% from newly synthesized fatty acids. Mn^{2+} again proved inhibitory with a marked 20-30% increase in activity upon its deletion similar to the acetate labelling experiment. Mg^{2+} also appeared to have either little effect or possibly inhibitory in the presence of Mn^{2+}. These cations have been shown not to be required for MGDG synthesis in spinach chloroplast

envelopes but are slightly beneficial individually (Heemskerk *et al.*, 1987).

Effects of Exogenous DAG Additions

DAG is a co-substrate required for galactolipid synthesis from UDP-galactose. All previous experiments described in this thesis rely on the endogenous supplies of DAG within the plastid membranes for galactolipid biosynthesis. However, it was of some interest to determine if exogenously supplied DAG could promote galactolipid synthesis and whether or not the effects were molecular species dependent. Because of the solubility problems of DAG's in aqueous media, detergents are frequently used to help disperse lipid substrates, with Tween commonly used in such studies of galactolipid synthesis (Mudd *et al.*, 1969; Marechal *et al.*, 1994). Unfortunately, the results obtained from one experiment to another were relatively variable, owing perhaps to the different amounts of plastid protein used in each experiment and thus different ratios of detergent:lipid:protein. Further, Tween itself was found to be greatly inhibitory. Nevertheless, in the presence of Tween, $(18:1)_2$-DAG*sn* consistently improved galactolipid biosynthesis by up to 3-fold (Table 6). The *sn* (or L) isoform of DAG appeared to give more consistent results than the racemized mixture of isoforms. The saturated forms were less stimulatory while other forms of DAG (i.e. 14:0) had

Table 5. The effect of cofactor deletion on galactolipid synthesis in pea root plastids using labelled UDP-galactose. Incubations contained 0.2 mM acetate, exp.#1 contained 0.011mM UDP-^{14}C-galactose (46μCi/μmole) and exp.#2 contained 0.077mM UDP-^{14}C-galactose (6.3μCi/μmole). (numbers are percentage of control, an average of two replicates per experiment)

	0.011mM	0.077mM
control*	100	100
-acetate	105	102
-ATP	70	81
-$MgCl_2$	98	118
-$KHCO_3$	82	98
-$MnCl_2$	122	131
-CoA	83	81
-NADH,NADPH	120	107

The % galactolipid distribution within each treatment showed little fluctuation with cofactor deletion. For MGDG the average was 94.31% (ranging from 90-99%), for DGDG 3.33% (ranging from 1-9%), while the residue left at the origin was 2.37% (ranging from 1-8%).

* Control activity for exp. #1 was 0.453 nmoles/hr/mg.
  Control activity for exp. #2 was 4.652 nmoles/hr/mg.

Table 6. The effect of exogenously supplied diacylglycerol on galactolipid synthesis in pea root plastids using labelled UDP-galactose. Incubations contained 0.2mM acetate and 11μM UDP-^{14}C-galactose (6.3μCi/μmole). (percentage of control with Tween)

treatment			percentage of control
control	+	0.03% Tween	100, 100, 100
control	+	0 Tween	854, 484
$(18:1)_2$DAG*sn*	+	0 Tween	889
$(18:1)_2$DAG*sn*	+	0.015% Tween	295
$(18:1)_2$DAG*sn*	+	0.03% Tween	146, 273, 280
$(18:0)_2$DAG*rac*	+	0.03% Tween	129
$(16:0)_2$DAG*rac*	+	0.03% Tween	129, 88
$(16:0)_2$DAG*sn*	+	0.03% Tween	73, 88
$(14:0)_2$DAG*rac*	+	0.03% Tween	103

Numbers represent individual experiments, diacylglycerol additions were 100 ug. Each number represents a separate experiment with different amounts of total membrane enzymes. All percentages are compared to their own individual controls (set to 100). Control activities ranged from 0.123 to 0.398 nmoles/hr/mg.

little effect. $(16:0)_2$-DAG*sn* had a slight inhibitory effect.

Several experiments have been conducted by other workers using exogenously applied DAG to investigate galactolipid synthesis. Mudd *et al.* (1969) used a similar concentration of Tween as used here (finding the direct application of DAG as too variable). They found that the greatest stimulation was by unsaturated DAG species. Joyard and Douce (1993) found that Triton at 0.9% was a strong inhibitor of DGDG formation. Caux and Weinberger (1993), in contrast, found that the concentration of Triton X-100 applied to *Lemna minor* plants could increase the total galactolipid content as well as the level of unsaturation in these plants. These observations have promoted some concern as to the relevance of such experimental methods to the conditions *in vivo*.

Marechal *et al.* (1994) appear to have been the most thorough in attempting to mimic the environment of the envelope. They found MGDG synthesis in spinach chloroplast membranes was affected differently by different species of DAG. The highest stimulation was with $(18:2)_2$-DAG*sn*. Although 18:2 was not tried in the experiments presented here, the unsaturated 18:1 was the most stimulatory. Similar *in vivo* results were reported by Williams *et al.* (1988) with $(^{14}C)CO_2$ treated leaves. He showed that 18:3 plants preferred

unsaturated DAG precursors. Marechal *et al.* (1994) also questioned the accessibility of exogenously supplied DAG in previous experiments by Mudd *et al.* (1969) and Heemskerk *et al.* (1987). In their kinetic paper on MGDG synthase Marechal *et al.* (1994) focused on DAG solubility and turbidity. Their results suggest that the production of MGDG can occur only when both UDP-galactose and DAG are coupled with the enzyme. They found that PG (an anionic phospholipid) and CHAPS (a zwitterionic detergent) where important to form an isotropic solution for mixed micellar addition of DAG to spinach chloroplast membranes. They felt this created an environment similar to the inner envelope membrane where MGDG synthesis is located in spinach chloroplasts.

CONCLUDING REMARKS

For the first time, it has been shown that pea root plastids are capable of MGDG synthesis in amounts comparable to chloroplasts. This ability is present even after root differentiation although these are nonphotosynthetic, nongreening plastids from an 18:3 plant. It has also been shown that the pathway for MGDG synthesis in pea root plastids (*in vitro*) is similar to 16:3 plants (FFA's to PA to DAG to MGDG) contrary to the idea that plastids (particularly chloroplasts) from 18:3 plants are thought to have insufficient PA phosphatase to support this activity.

The synthesis of MGDG in these plastids occurred only upon the addition of UDP-galactose. Total incorporation of labelled acetate into lipid was stimulated by 60% when UDP-galactose was added to a concentration of 75μM. This suggests UDP-galactose has an affect on the enzymes involved in fatty acid and ultimately glycerolipid production. Further studies on these enzymes are needed to characterize this phenomena. After a one hour incubation 30% of the label occurred in MGDG. MGDG synthesis caused a corresponding decrease in DAG upon the addition of UDP-galactose thus confirming the known pathway for galactolipid synthesis (figure 2). DGDG was generally not detectable

(<2% of radiolabelled incorporation) except when detection was forced by high specific activity of the precursor.

The endogenous lipids, consistent with the thought of pea as an 18:3 plant, contained 80% C_{18} in the fatty acids of MGDG, DGDG, TG and PC. However, in labelled acetate experiments palmitate was the predominately labelled fatty acid in all lipids except PC (where 80% was 18:1). The precursors PA and DAG had similar ratios of 16:0-18:0-18:1 to that of MGDG. Positional analysis showed that most of the label was associated with the *sn-2* position suggesting use of endogenous lyso-PA or involvement of acyl exchange.

The cofactors absolutely required for fatty acid synthesis were not the same as those required for galactolipid synthesis. Using labelled acetate, the total incorporation decreased by as much as 86% when the cofactors bicarbonate, CoA or reduced nucleotides were omitted from the incubation medium. However, the % incorporation into MGDG increased suggesting a preferential channelling of fatty acids into galactolipids. Experiments using UDP-^{14}C-galactose instead of acetate showed that a reduction of only 20% occurred upon the deletion of ATP, bicarbonate or reduced nucleotides. This suggests that under the conditions used here, only 20% of galactolipid synthesis involves newly synthesized fatty acids. Galactolipid synthesis therefore relies primarily on

endogenous pools of DAG's and acyl exchange.

The cations Mg^{2+} and Mn^{2+} were not as required as for fatty acid and glycerolipid synthesis in pea root plastids (Stahl, 1990; Xue, 1993). Using labelled UDP-galactose, the deletion of Mg^{2+} caused little variation from the control while a stimulation of incorporation was achieved with the deletion of Mn^{2+}. This suggests the cations in combination (at the levels used here) may be inhibitory.

The addition of Tween was inhibitory. However, with the addition of $(18:1)_2$-DAG*sn* galactolipid synthesis was improved threefold. This is noteworthy considering these plastids contain both eukaryotic and prokaryotic pathways and do not fit the conventional thoughts of 16:3 vs. 18:3 plants. Further studies on PC's influence and regulation in conjunction with the eukaryotic pathway (ie the use of C_{18} in the *sn-2* position) could prove useful for a more complete understanding of root plastidic lipid metabolism.

The production of MGDG has now been demonstrated in pea root plastids but further experiments are needed to determine the extent possible for DGDG synthesis. Changes in pH, temperature and ionic content of the media as used in chloroplast studies (Heemskerk *et al.*, 1987) may prove helpful. Changes in temperature could also influence the

channelling of DAG to TAG as well as the level of desaturation of galactolipids. Similar studies, with the appropriate additions (SO_4 or APS), could also affect the production of SQDG from DAG.

It would be interesting to compare these plastids to greening ones through changes in light regimes as well as specific changes known to occur upon photosynthesis (ie ionic concentrations including Mg^{2+}). Upon envelope isolation the location, kinetic and regulatory properties of the enzymes MGDG and DGDG synthase could further determine the pathway requirements. Isolation and characterization of these enzymes could prove potential tools for gene isolation and eventual genetic manipulation of the pathway. This could prove useful in such endeavors as reducing chilling and ozone injury.

This research has advanced the characterization of galactolipid synthesis in roots. Further work is still required to fully characterize the mode of regulation of higher plant lipid metabolism in non-photosynthetic plastids.

LITERATURE CITED

Alban C, Joyard J, Douce R (1988) Preparation and characterization of envelope membranes from nongreen plastids. Plant Physiol 88:709-717

Alban C, Dorne AJ, Joyard J, and Douce R (1989)[^{14}C]Acetate incorporation into glycerolipids from cauliflower proplastids and sycamore amyloplasts. FEBS Lett 249(1):95-99

Alban C, Joyard J, Douce R (1989) Comparison of glycerolipid biosynthesis in non-green plastids from sycamore (*Acer pseudoplatanus*) cells and cauliflower (*Brassica oleracea*) buds. Biochem J 259:775-783

Alberdi M, and Corcuera LJ (1991) Cold acclimation in plants. Phytochem 30:3177-3184

Alberdi M, Corcuera LJ, Maldonado C, Barrientos M, Fernandez J, and Henriquez O (1993) Cold acclimation in cultivars of *Avena sativa*. Phytochem 33:57-60

Bakaltcheva I, Williams W.P., Schmitt JM, and Hincha DK (1994) The solute permeability of thylakoid membranes is reduced by low concentrations of trehalose as a co-solute. Biochim Biophys Acta 1189:38-44

Bertrams M, Wrage K, and Heinz E (1981) Lipid labelling in intact chloroplasts from exogenous nucleotide precursors. Z Naturforsch 36c:62-70

Bishop DG, Sparace SA, and Mudd JB (1985) Biosynthesis of sulfoquinovosyldiacylglycerol in higher plants: The origin of the diacylglycerol moiety. Arch Biochem Biophys 240:851-858

Browse J, McCourt PJ, and Somerville CR (1986) Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal Biochem 152:141-145

Browse J, Somerville C (1991) Glycerolipid synthesis: Biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol 42:467-506

Caux PY, and Weinberger P (1993) *Lemna minor* membranes affected by adjuvants. Environ Pollut 81:151-156

Carlsson AS, Hellgren LI, Sellden G, and Sandelius AS (1994) Effects of moderately enhanced levels of ozone on the acyl lipid composition of leaves of garden pea (*Pisium sativum*). Physiologia Pl 91:754-762

Carter HE, McCluer RH, Slifer ED (1956) Lipids of wheat flour. I. Characterization of galactosylglycerol components. J Am Chem S 7:3735- 3738

Cho Sh (1993) Effects of dark treatment and DCMU on desaturation of galactolipids in *Dunaliella salina*. Korean J Bot 36:293-296

Chupin V, Van't Hof R, and De Kruijff B (1994) The transit sequence of a chloroplast precursor protein reorients the lipids in monogalactosyl diglyceride containing bilayers. FEBS Lett 350:104-108

Cline K, and Keegstra K (1983) Galactosyltransferases involved in galactolipid biosynthesis are located in the outer membrane of pea chloroplast envelopes. Plant Physiol 71:366-372

Coves J, Block MS, Joyard J, and Douce R (1986) Solubilization and partial purification of UDP-galactose:diacylglycerol galactosyltransferase activity from spinach chloroplast envelope. FEBS Lett 208:401-406

Coves J, Joyard J, and Douce R (1988) Lipid requirement and kinetic studies of solubilized UDP-galactose:diacylglycerol galactosyltransferase activity from spinach chloroplast envelope membranes. Proc Natl Acad Sci U.S.A. 85:4966-4970

Dalgarn D, Miller P, Bricker T, Speer N, Jaworski JG, Newman DW (1979) Galactosyl transferase activity of chloroplast envelopes from senescent soybean cotyledons. Plant Sci L 14:1-6

Dominquez C, Tena M, Rodriquez I, and De Prado R (1994) Lipid composition and chloroplast ultrastructure in triazine resistant biotypes of *Amaranthus* species. Plant Physiol 143:637-642

Dorne AJ, Block MA, Joyard J, Douce R (1982) The galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer membrane of the chloroplast envelope. FEBS Lett 145:30-34

Douce R(1974) Site of biosynthesis of galactolipids in spinach chloroplasts. Sci 183:852-853

Douce R, and Joyard J (1980) Plant galactolipids, *In:* The Biochemistry of Plants, Vol. 4, Lipids: Structure and Function, Stumpf, PK, Ed, Academic Press, NY,pp 321-362

Fishwick MJ, and Wright AJ (1980) Isolation and characterization of amyloplast envelope membranes from *Solanum tuberosum*. Phytochem 19:55

Frentzen M, Heinz E, Mckeon T, and Stumpf PK (1982) De novo-biosynthesis of galactolipid molecular species by reconstituted enzyme systems from chloroplasts. *In:* Biochemistry and Metabolism of Plant Lipids. JFGM Wintermans and PJC Kuiper,Eds., Elsevier, Amsterdam. pp.141-151

Fuks B, and Homble F (1994) Permeability and electrical properties of planar lipid membranes from thylakoid lipids. Biophys J 66:1404-1414

Gounaris K, Barber J, and Harwood JL (1986) The thylakoid membranes of higher plant chloroplasts. Biochem J 237:313-316

Grant CWM, and Peters MW (1984) Lectin-membrane interactions information from model systems. Biochim Biophys Acta 779:403-422

Harwood JL (1989) Lipid Metabolism in plants. Crit. Rev. Plant Sci 8:1-43

Harwood JL, and Russell NJ (1984) Lipids in plants and microbes. George Allen & Unwin, London. 162 pp.

Heemskerk JWM, Jacobs, FHH, Scheijen MAM, Helsper JPFG, Wintermans JFGM (1987) Characterization of galactosyltransferases in spinach chloroplast envelopes. Biochim Biophys Acta 918:189-203

Heemskerk JWM, Jacobs FHH, Wintermans JFGM (1988) UDPgalactose-independent synthesis of monogalactosyl-diacylglycerol. An enzymatic activity of the spinach chloroplast envelope. Biochim Biophys Acta 961:38-47

Heemskerk JWM, Schmidt H, Hammer U, and Heinz E (1991) Biosynthesis and desaturation of prokaryotic galactolipids in leaves and isolated chloroplasts from spinach. Plant Physiol 96:144-152

Heemskerk JWM, Storz T, Schmidt RR, and Heinz E (1990) Biosynthesis of digalactosyldiacylglycerol in plastids from 16:3 and 18:3 plants. Plant Physiol 93:1286-1294

Heemskerk JWM, Wintermans JFGM, Joyard J, Block MA (1986) Localization of galactolipid: galactolipid galactosyltransferase and acyltransferase in outer envelope membrane of spinach chloroplasts. Biochim Biophys Acta 877:281-289

Heinz E (1977) Enzymatic reactions in galactolipid biosynthesis, *In*: Lipids and Lipid Polymers, Tevini M and Lichtenthaler HK, Eds, Springer-Verlag, Berlin, pp102-120

Heinz E, and Roughan PG (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol 72:273-279

Hincha DK, Bakaltcherva I, and Schmitt JM (1993) Galactose-specific lectins protect isolated thylakoids against freeze-thaw damage. Plant Physiol 103: 59-65

Jamieson GR, and Reid EH (1971) The occurrence of hexadeca-7,9,13-trienoic acid in the leaf lipids of angiosperms. Phytochem 10:1837-1843

Jaworski JG (1987)Biosynthesis of monoenoic and polyenoic fatty acids, *In:* The Biochemistry of Plants, Vol.9, Lipids: Structure and Function, Stumpf PK, Ed., Academic Press, New York, pp159-174

Journet EP, and Douce R (1985) Enzymatic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. Plant Physiol 79:458

Joyard J, Block MA, Malhere A, Marechal E, and Douce R. (1993) Origin and Synthesis of Galactolipid and Sulfolipid Head Groups in Lipid Metabolism in Plants, Moore S, Ed., CRC Press, Florida. 231-258

Joyard J, and Douce R (1987) Galactolipid synthesis *In:* The biochemistry of plants. A comprehensive treatise. Stumpf PK, and Conn EE, Eds, Vol 9. Lipids: Structure and Function, Stumpf PK, Ed. pp 215-274

Kaniuga Z, and Gemel J (1984) Galactolipase activity and free fatty acid levels in chloroplasts, novel approach to characteristics of chilling sensitivity of plants. FEBS Lett 171:55-58

Kleinig J, and Liedvogel B (1978) Fatty acid synthesis by isolated chromoplasts from the daffodil:[^{14}C]acetate incorporation and distribution of labelled acids. Eur J Bioch 83:499

Kleppinger-Sparace KF, Mudd JB, and Bishop DG (1985) Biosynthesis of sulfoquinovosyldiacylglycerol in higher plants: The incorporation of $^{35}SO_4$ by intact chloroplasts. Arch Biochem Biophys 240:859-865

Kleppinger-Sparace KF, Stahl RJ, and Sparace SA (1992) Energy requirements for fatty acid and glycerolipid biosynthesis from acetate by isolated pea root plastids. Plant Physiol 98:723-727

Konigs B, and Heinz E. (1974)Investigation of some enzymatic activities contributing to the biosynthesis of galactolipid precursors in *Vicia faba*. Planta 118:159-169

Lapage M (1967) Identification and composition of turnip root lipids. Lipids 2:244-250

Leech RM, Rumsby MG, and Thomson WW (1973) Plastid differentiation, acyl lipid, and fatty acid changes in developing green maize leaves. Plant Physiol 52:240-245

Lem NW, and Williams JP (1983) Changes in the ^{14}C-labelling of molecular species of 3-monogalactosyl-1,2-diacylglycerol in leaves of *Vicia faba* treated with compound San 9785. Biochem J 209:513-518

Liedvogel B, and Kleinig H (1979) Galactolipid synthesis in chromoplasts in vitro. Planta 144:467-471

Liedvogel B, Kleinig H, Thompson JA, and Falk H (1978) Chromoplasts of *Tropaeolum majus* L.: Lipid synthesis in whole organelles and subfractions. Planta 141:303-309

Lowry OH, Rosenbrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 192:265-275

Lyons JM, Raison JK, and Steponkus PL (1979) in: Low Temperature Stress in Crop Plants. The Role of the Membrane. Lyons, JM *et al.*,Eds., Academic Press, New York pp 1-24

Marechal E, Block MA, Joyard J, and Douce R (1991) Purification de l'UDP-galactose:1,2-diacylglycerol galactosyltransferase de l'enveloppe des chloroplastes d'epinard. Acad Sci (Paris) CR 313:521-528

Marechal E, Block MA, Joyard J, and Douce R. (1994) Kinetic properties of monogalactosyldiacylglycerol synthase from spinach chloroplast envelope membranes. J Biol Chem 269:57[illegible]-5798

Menikh A, and Fragata M (1993) Fourier transform infrared spectroscopic study of ion binding and intramolecular interactions in the polar head of digalactosyl-diacylglycerol. Eur Biophys J 22:249-258

Miquel M, and Dubacq JP (1992) In situ incorporation of fatty acids into lipids of the outer and inner envelope membranes of pea chloroplasts. Plant Physiol 100:472-481

Morre DJ, Morre JT, Morre SR, Sundqvist C, and Sandelius AS (1991) Chloroplast biogenesis. Cell-free transfer of envelope monogalactosylglycerides to thylakoids. Biochim Biophys Acta 1070:437-445

Mudd JB, Andrews JE, Sanchez J, Sparace SA, Kleppinger-Sparace K (1985) Biosynthesis of chloroplast lipids.*In:* Frontiers in Membrane Research in Agriculture, St. John JB, Berlin E, and Jackson PC, Eds., Rowman and Allenheld, Totowa pp 36-53

Mudd JB, Van Vliet HHDM, and Van Deenen LLM (1969) Biosynthesis of galactolipids by enzyme preparations from spinach leaves. J Lipid Res 10:623-630

Murphy DJ (1982) The importance of non-planar bilayer regions in photosynthetic membranes and their stabilisation by galactolipids. FEBS Lett 150:19-26

Nakamura Y, and Yamada M (1975) Fatty acid synthesis by spinach chloroplasts. I. Property of fatty acid synthesis from acetate. Plant Cell Physiol 16:139

Nussberger S, Doerr K, Wang DN, and Kuhlbrandt W (1993) Lipid-protein interactions in crystals of plant light-harvesting complex. J Mol Biol 234:347-356

Ohlrogge JB, Browse J, and Somerville CR (1991)The genetics of plant lipids. Biochim Biophys Acta 1082:1-26

Paczkowski C, and Wojciechowski ZA (1994) Glucosylation and galactosylation of diosgenin and solasodine by soluble glycosyltransferases(s) from *Solanum melongena* leaves. Phytochem 35(6):1429-1434

Polashock JJ, Chin CK, and Martin CE (1992) Expression of the yeast delta-9 fatty acid desaturase in *Nicotiana tabacum*. Plant Physiol 110(2):894-901

Qi Q, and Sparace SA (1994) unpublished observations.

Qi Q, Kleppinger-Sparace KF, and Sparace SA (1995) The utilization of glycolytic intermediates as precursors for fatty acid biosynthesis by pea root plastids. Plant Physiol 107:413-419

Quinn PJ, and Williams WP (1983) The structural role of lipids in photosynthetic membranes. Biochim Biophys Acta 737:223-266

Quoc KP, Dubacq JP, Demandre C, and Mazliak P (1994) Comparative effects of exogenous fatty acid supplementations on the lipids from the cyanobacterium *Spirulina platensis*. Plant Physiol Biochem 32:501-509

Rais LB, Alpha MJ, Khahl J, Guillot-Salomon T, and Dubacq JP (1993) Lipid and protein contents of jojoba leaves in relation to salt adaptation. Plant Physiol Biochem (Paris) 31:547-557

Rathmore DS, Kumar A, and Kumar HD (1993) Lipid content and fatty acid composition in nitrogen-fixing cyanobacterium *Anabaena doliolum* as affected by molybdenum deficiency. World J Microbiol Biotechnol 9:508-510

Rawyler A, Meylan M and Siegenthaler PA (1992) Galactolipid export from envelope to thylakoid membranes in intact chloroplasts. I. Characterization and involvement in thylakoid lipid asymmetry. Biochim Biophys Acta 1104:331-341

Roughan PG (1975) Phosphatidyl choline: Donor of 18-carbon unsaturated fatty acids for glycerolipid biosynthesis. Lipids 10:609-614

Roughan PG (1987) On the control of fatty acid compositions of plant glycerolipids, *In:* The Metabolism, Structure and Function of Plant Lipids, Stumpt PK, Mudd JB, Nes WD, Eds. Plenum Press, NY, pp247

Roughan PG, and Slack R (1984) Glycerolipid synthesis in leaves. Trends Biochem Sci 9:383-386

Saczynska V, Gemel J, and Kaniuga Z (1993) Chilling susceptibility of *Cucumis sativus* species. Phytochem 33:61-67

Sakaki T, Tanaka K, and Yamada M (1994) General metabolic changes in leaf lipids in response to ozone. Plant Cell Physiol 35:53-62

Sakaki T, Saito K, Kawaguchi A, Kondo N, and Yamada M (1990) Conversion of monogalactosyldiacylglycerols to triacylglycerols in ozone-fumigated spinach leaves. Plant Physiol 94:766-772

Sanchez J, and Mudd JB (1993) Bicarbonate stimulates stearate synthesis in chloroplasts: Discrimination against stearate in the biosynthesis of glycerolipids. Phytochem 34:1021-1027

Siebertz HP, and Heinz E (1977) Galactosyldiacylglycerols by cell-free preparations from pea leaves. Hoppe Seyler's Z Physiol Chem 358:27-34

Somerville C, and Browse B (1991) Plant lipids: Metabolism, mutants, and membranes. Sci 252:80-87

Sparace SA, and Kleppinger-Sparace KF (1993) Metabolism in nonphotosynthetic, nonoilseed Tissues *In* Lipid Metabolism in Plants, Moore TS, Ed. CRC Press, Florida. pp569-589

Sparace SA, Kleppinger-Sparace KF, Stahl RJ, Xue L, and Qi Q (1993) Lipid Biosynthesis in Pea Root Plastids and Some Effects of Glycolytic Intermediates in Seed Oils for the Future, Mackenzie SL, Taylor DC, Eds, AOCS Press, Illinois, pp52-60

Sparace SA, and Mudd JB (1982) PG synthesis in spinach chloroplasts:characterization of the newly synthesized molecule. Plant Physiol 70:1260-1264

Stahl RJ (1990) Fatty acid and glycerolipid biosynthesis in pea root plastids. M.Sc. thesis, Department of Plant Science,McGill University

Stahl RJ, and Sparace SA (1991)Characterization of fatty acid biosynthesis in isolated pea root plastids. Plant Physiol 96:602-608

Stumpf PK (1984) Fatty acid biosynthesis in higher plants, in Fatty Acid Metabolism and Its Regulation, Numa S, Ed., Elsevier, Amsterdam, pp155

Terzaghi WB (1989) Manipulating membrane fatty acid compositions of whole plants with tween-fatty acid esters. Plant Physiol 91:203-212

Teucher T, and Heinz E (1991) Purification of UDP-galactose:diacylglycerol from chloroplast envelopes of spinach (*Spinacia oleraea* L.). Planta 184:319-326

Tremolieres A, Dainese P, and Bassi R (1994) Heterogenous lipid distribution among chlorophyll-binding proteins of photosystem II in maize mesophyll chloroplasts. European J Biochem 221:721-730

Tremolieres A, and Lepage M (1971) Changes in lipid composition during greening of etiolated pea seedlings. Plant Physiol 47:329-334

Van Besouw A, and Wintermans JFGM (1978) Galactolipid formation in chloroplast envelopes I. Evidence for two mechanisms in galactosylation. Biochim Biophys Acta 529:44-53

Van Besouw A, and Wintermans JFGM (1979) The synthesis of galactolipids by chloroplast envelopes. FEBS Lett 102:33-47

Webb MS, and Green BR (1991) Biochemical and biophysical properties of thylakoid acyl lipids. Biochim Biophys Acta 1060:133-158

Wellburn AR, Robinson DC, Thomson A, and Leith ID (1994) Influence of episodes of summer O-3 on DELTA-5 and DELTA-9 fatty acids in autumnal lipids of Norway spruce (*Picea abies* L. Karst). New Phytol 127(2):355-361.

Williams JP, Khan MU, and Mitchell K (1988) The molecular species of diacylglycerol precursors used in monogalactosyl-diacylglycerol biosynthesis in the leaves of a number of higher plant species. Plant Cell Physiol 29:849-854

Wintermans JFGM (1960) Concentrations of phosphatides and glycolipids in leaves and chloroplasts. Biochim Biophys Acta 44:49

Xue L (1993) Glycerolipid biosynthesis in pea root plastids. M. Sc. Thesis, Deptartment of Plant Science, McGill University

Zhao KF, Lue YF, Zhang BZ, and Yi JL (1993) Influence of calcium on alleviating sodium chloride-induced injury effects in wheat seedlings. Acta Bot Sin 35:51-56