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FATTY ACID METABOLISM IN HIGHER PLANTS:
PROBING THE MECHANISM OF FATTY ACID TRANSPORT
ACROSS THE CHLOROPLAST ENVELOPE

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Major Professor's Signature

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By

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ABSTRACT

FATTY ACID METABOLISM IN HIGHER PLANTS:
PROBING THE MECHANISM OF FATTY ACID TRANSPORT ACROSS THE CHLOROPLAST ENVELOPE

By

Abraham Jeong-Kyu Koo

Fatty acids (FA's) play a variety of crucial roles in living organisms. Despite the important roles in various aspects of life, FA transport remains one of the most poorly understood areas of FA metabolism. The main objective of this thesis is to begin to determine the mechanism(s) of FA transport in plants. Lipid metabolism within the cell requires massive movements of acyl chains between intracellular organelles. One such example is the export of FA from the plastid. *De novo* fatty acid synthesis (FAS) in plants occurs almost exclusively in the plastids. The free fatty acid (FFA) released at the final step of FAS by hydrolysis of acyl-acyl carrier protein (ACP) crosses the double envelope membrane to be re-esterified to acyl-coenzyme A in the cytosol. The maximum half life for FFA in this export pool in pea and spinach leaves was determined to be ≤ 1 s. Kinetic labeling experiments with isolated pea chloroplasts indicated that the measured long-chain acyl CoA synthesis (LACS) reaction using freely diffusing bulk FFA substrate cannot account for the efficient LACS reaction that must occur at the very low *in vivo* concentrations of FFA. The "nascent" FFA was also shown to be protected from binding to extraplastidial bovine serum albumin before being converted to the long-chain acyl CoA. These data discount a free diffusion model of FFA export and indicate a channeled delivery of FFA to the LACS.

Plant cells are capable of incorporating, elongating and desaturating exogenously provided short- and medium-chain FA's. The existence of a pathway in the plastid for the esterification of exogenous FFA to ACP has only been inferred from past studies. In this thesis the plastid was demonstrated to be the major site for the elongation of the exogenous FFA into C16 and C18 FA's. An Arabidopsis T-DNA insertion mutant, *aae15,* was isolated through a reverse genetics approach, and this mutant displayed as much as 80% reduction in elongation of exogenous [1-^{14}C]laurate to C16 and C18 FA's. The gene (*AAE15* (acyl activating enzyme15)) disrupted in the mutant is a homolog of long-chain acyl-CoA synthetases, is represented by 11 expressed sequence tags (EST's) and contains a plastid targeting sequence. The findings indicate the existence of a novel pathway of FFA metabolism in the plastid.

It is likely that specific membrane proteins are involved in lipid traffic across the envelope. Despite the importance and complexity of the plastid envelope, only a small fraction of integral envelope proteins have been purified or characterized. Through a bioinformatics strategy 541 proteins were selected from the *Arabidopsis thaliana* nuclear genome as potential candidates of the plastid inner envelope membrane proteins (*At*PEM candidates). A web based database was developed with detailed information about the predicted proteins, including their expression profiles based on EST databases. Twenty ATP Binding Cassette transporter proteins were selected as candidates for plastid envelope lipid transporters and 11 T-DNA insertion mutants were analyzed for FA compositions and contents. The knock-outs of At1g70610 and At5g64940 consistently showed reduced oil contents (about 50-70% of wild type) in seeds and therefore are candidates for further analysis as potential lipid transporters.

The LORD is my shepherd, I shall not be in want.

Psalm 23:1

To

My grandfather &

Joseph Hong (PhD candidate) &

Hester Hughes (PhD candidate)

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CHAPTER 1

INTRODUCTION

Fatty acids play a variety of roles in living organisms (see Table 1). Fatty acids are the core components of all biological membranes. Fatty acids are a crucial energy source for animals especially in heart muscles, skeletal muscles, liver and adipose tissue. In plants fatty acids are the most important means to store carbon and energy for the growth of the next generation. Fatty acids are also the most abundant form of reduced carbon chains available from nature and have diverse uses ranging from food to industrial feedstocks. Current world vegetable oil production is estimated at 87 million metric tons with an approximate market value of 40 billion U.S. dollars (Gunstone, 2001). As much as 25% of human caloric intake in developed countries is derived from plant fatty acids (Broun et al, 1999; Thelen and Ohlrogge, 2002). Plant fatty acids are also the major ingredients of nonfood products such as soaps, detergents, lubricants, biofuels, cosmetics, and paints (Ohlrogge, 1994).

The basic metabolic pathways leading to the production of fatty acids (FA's) and their major derivatives have been well studied (reviewed below) and are known to occur by similar mechanisms in all organisms. However, there is one major area that has failed to be explored in lipid research and that area is the various aspects of fatty acid movement. Comparatively more effort has been put into understanding fatty acid transport issues in animal systems largely due to their important medical implications. However, until recently there has essentially been no major research done in plants on this topic. The

work described in this dissertation is one of the few pioneering studies dealing with fatty acid transport in plants (Footitt et al., 2002; Xu et al., 2003). The main objective of this thesis is to begin to explore the mechanism(s) of fatty acid transport in plants. I begin this thesis with the following goals:

1. Understand the general topic and issues of fatty acid movement.

2. Examine and define research areas to be explored in plants in terms of FA transport.

3. Gain new information on at least one major aspect of fatty acid transport and develop strategies for the future research directions.

Table 1. Functions of fatty acid derived lipids in higher plants.

Function	**FA derived lipids**	**references**
Membrane structural components	glycerolipids, sphingolipids	Browse and Somerville, 1994
Storage compounds	triacylglycerols, waxes	Ohlrogge and Browse, 1995
Surface protection and water proofing	cutin, suberin, surface waxes	Kunst and Samuels, 2003
Signaling	jasmonate, oxylipins, diacylglycerol, phosphatidylinositol 4,5-bisphosphate, phosphatidic acid	Vick and Zimmerman, 1984; Farmer and Ryan, 1990; Alexandre and Lassalles, 1992; Cote and Crain, 1993; Munnik, 2001
Posttranslational protein modification	myristic acid, palmitic acid, phosphatidylinositol, ceramide	Towler et al., 1988; McIlhinney, 1990; Milligan et al., 1995

Chapter I is organized to give an overview of 1) FA metabolism in plants with an emphasis on the subject of FA movements, 2) the debate over FA membrane transport in animal and other cells and 3) the major lipid transporter candidates identified in various organisms. I devote a relatively large part of chapter I to describe studies in other (non-plant) organisms because so little information is available for plants.

Fatty Acid Metabolism in Higher Plants: the underlying questions about FA movements

Unlike animal cells, each plant cell is autonomous with respect to fatty acid synthesis, and there is believed to be no extensive exchange of fatty acids or lipids between cells and between distant parts of plant tissues. However, lipid metabolism within the cell demands movement of fatty acids or lipids from one organelle to another. Transport systems control the flux of metabolites between organelles, and are often highly regulated. This may be the case for fatty acids, representing as they do the key building blocks for membrane lipid biogenesis.

The majority of the de novo fatty acid synthesis product must be exported from the plastid

In plants, *de novo* fatty acid synthesis (FAS) occurs almost exclusively in the plastid (Ohlrogge et al., 1979). FAS is a continued cycle of reactions that result in growth of fatty acyl chain by 2 carbon units (Figure 1). The source of the 2 carbon units are derived from acetyl-CoA which becomes committed to FAS by the action of acetyl-CoA carboxylase (ACCase) which catalyses the ATP dependent carboxylation of acetyl-CoA to yield malonyl-CoA (Ohlrogge and Browse, 1995). Acyl carrier protein (ACP) is used as a scaffold of the growing acyl chain and used as a vehicle to move FA chains between

sequential biosynthetic steps. Except for the primary condensation reaction between malonyl-ACP and acety-CoA, the elongation reaction proceeds by condensation between the growing chain of acyl-ACP and malonyl-ACP. The condensation reaction is catalyzed by a class of enzymes called β-ketoacyl-ACP synthases (KAS). Generally in higher plants these cycles continue seven or eight rounds forming 16 or 18 carbon saturated acyl-ACP's. The elongation reaction stops when the growing FA chain is either hydrolyzed to a free fatty acid molecule or alternatively transferred to another acceptor molecules (glycerol-3-phosphate, lysophosphatidic acid) to form lyso-/phosphatidic acid for plastidial glycerolipid synthesis.

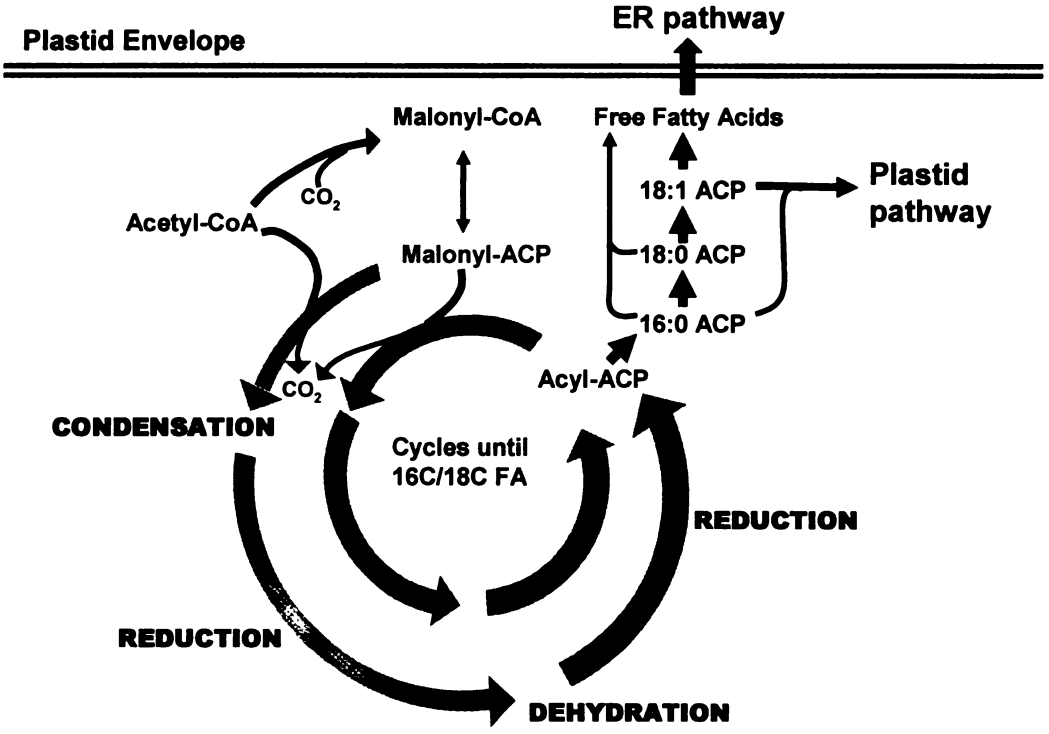
Problem

Since the plastid is the primary and almost the only source of fatty acid precursors for the entire cell, the flux of acyl chains through the plastid envelope should be large. In addition fatty acid synthesis and utilization must be well coordinated and regulated in order to meet the different demands for FA's in/at various cell types and developmental stages (Ohlrogge and Jaworski, 1997). It is not known how the plastid synthesized fatty acids get exported, nor how transport is regulated. The current model only proposes that the released free fatty acid crosses the double membrane of plastid envelope to the outer envelope where it is reactivated to acyl-CoA for utilization in cytosolic lipid synthesis. The basis of this model comes from the observations that the acyl-ACP thioesterase is a stromal activity (Shine et al., 1977; Doermann et al., 1995; Voelker et al., 1997), while the acyl-CoA synthetase is associated with the outer envelope (Block et al., 1983; Andrews and Keegstra, 1983). In addition free fatty acids are end-products of fatty acid

synthesis in chloroplast assays, unless ATP and CoA are added, in which case acyl-CoA becomes a major product (Roughan and Slack, 1982). The involvement of a hydrolytic step in this process was conclusively demonstrated by a study using *in vivo* ^{18}O labeling technique (Pollard and Ohlrogge, 1999). Chapter 2 of this thesis addresses the question whether the export of free fatty acid involves free physical diffusion or involves a channeled system.

Figure 1. Schemetic of FAS in the plastid.

The thickness of arrows does not necessarily indicate the flux.



Plant membrane lipid biosynthesis requires extensive exchange of lipids between organelles

Membrane glycerolipids have FA's attached to the sn-1 and sn-2 positions of the glycerol backbone and a polar head group in the sn-3 position. The combination of nonpolar acyl chains with polar head groups leads to the amphipathic physical properties of these molecules, which are essential for the formation of membrane bilayers (Nagle et al., 2000). The acyl chains usually are composed of 16 and 18 hydrocarbon chains with one to three *cis* double bonds in plants.

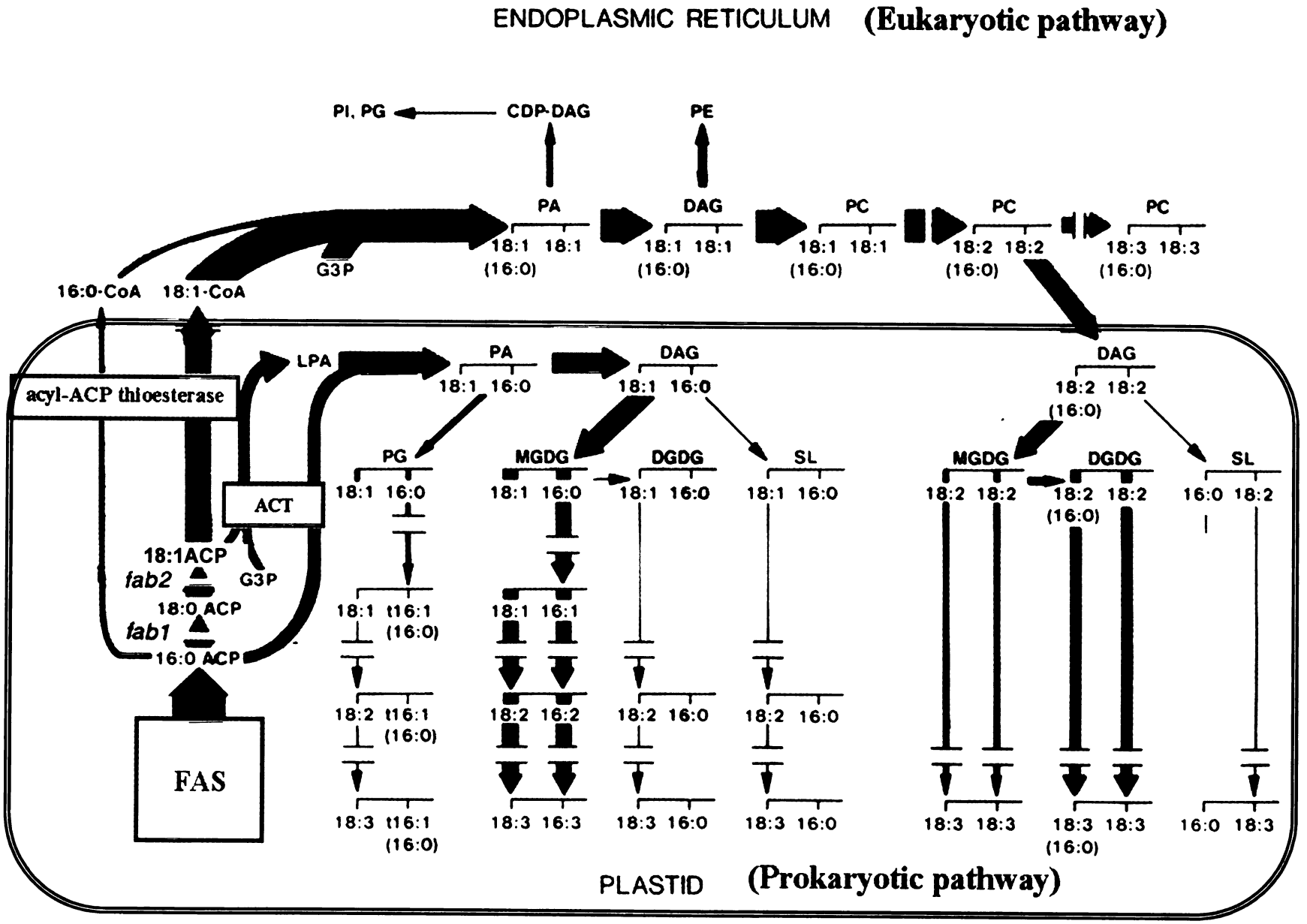
Two distinct pathways for glycerolipid synthesis exist in higher plants (Figure 2). Based on their similarity to the bacterial pathway and animal pathway they have been termed the prokaryotic pathway and the eukaryotic pathway respectively (Roughan and Slack, 1982). The prokaryotic pathway takes place within the chloroplast whereas the eukaryotic pathway involves export of free fatty acid from the plastid and glycerolipid synthesis in the endoplasmic reticulum (ER). Some of these ER synthesized lipids return back to the chloroplast for the synthesis of plastidial glycerolipids. The eukaryotic pathway is the principal route of glycerolipid synthesis in all nonphotosynthetic tissues as well as in the photosynthetic tissues of 18:3 plants. In leaves of 16:3 plants such as Arabidopsis and spinach both pathways contribute about equally to the synthesis of leaf glycerolipids (Somerville and Browse, 1991).

Problem

According to the two-pathway model, a considerable amount of lipid should travel back to plastids from the ER. In addition, evidence from several Arabidopsis mutants

Figure 2. Schematic overview of the glycerolipid biosynthetic pathways in Arabidopsis (adapted from Browse and Somerville, 1991).

Abbreviations for the lipid structures: G3P, glycerol-3-phosphate; LPA, 1-acyl-glycerol-3-phosphate; PA, phosphatidic acid; DAG, diacylglycerol; PG, phosphatidylglycerol; PG-P, phosphatidylglycerol-3-phosphate; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SL, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.


ENDOPLASMIC RETICULUM (Eukaryotic pathway)
PI, PG
CDP-DAG
PE
PA
DAG
PC
PC
PC
18:1 18:1 (16:0)
18:1 18:1 (16:0)
18:1 18:1 (16:0)
18:2 18:2 (16:0)
18:3 18:3 (16:0)
G3P
16:0-CoA
18:1-CoA
acyl-ACP thioesterase
LPA
PA
18:1 16:0
DAG
18:1 16:0
DAG
18:2 18:2 (16:0)
ACT
PG
MGDG
DGDG
SL
18:1 16:0
18:1 16:0
18:1 16:0
18:1 16:0
MGDG
DGDG
SL
18:2 18:2
18:2 18:2 (16:0)
16:0 18:2
18:1ACP
fab2
G3P
18:0 ACP
fab1
16:0 ACP
18:1 t16:1 (16:0)
18:1 16:1
18:2 t16:1 (16:0)
18:2 16:2
18:2 16:0
18:2 16:0
18:3 t16:1 (16:0)
18:3 16:3
18:3 16:0
18:3 16:0
18:3 18:3
18:3 18:3 (16:0)
16:0 18:3
FAS
PLASTID (Prokaryotic pathway)

indicates that lipid exchange between the ER and the plastid is somewhat reversible (Miquel and Browse, 1992; Browse et al., 1993). Mutants deficient in ER desaturases still contain polyunsaturated fatty acids in extraplastid membranes which suggests that they were derived from the plastid. Also, several lines of evidence indicate the existence of regulatory mechanisms that coordinate the activity of the two pathways. For example, the *act1* mutant of Arabidopsis is deficient in activity of the first enzyme of the prokaryotic pathway and thus has severely reduced flux through the prokaryotic pathway (Kunst et al., 1988). However the mutant plant compensates the deficiency by increasing the flux through the eukaryotic pathway. What are the signals that communicate between the ER and the plastid to coordinate these pathways? What is the system that the plant cells use to move hydrophobic lipid molecules between each membrane compartments? How does the DAG moiety coming from ER cross the outer membrane of the plastid envelope and reach the inner membrane where MGDG synthesis occurs? Vesicular transport between the ER and the plastid has not been observed. Once lipid-transfer proteins were thought to be the intracellular carriers of lipid molecules, but it has been shown that their main location is outside the plasma membrane in the cell wall space of epidermal cells (Bernhard et al., 1991; Sterk et al., 1991; Thoma et al., 1993).

The plastid envelope is clearly an intermediary site for exchange of both fatty acids and lipids. The major fluxes are believed to involve fatty acid export from plastids and glycerolipid import from the ER, although movements in the opposite direction may occur in some cases (Chapter 3; Browse et al., 1993). A large part of lipid metabolism also occurs at the inner and outer membranes of plastid. It is likely that specific

membrane proteins mediate the exchange of lipid molecules between ER and plastid. The transfer of lipid moieties between the outer envelope membrane and the inner envelope membrane of the plastid also is likely to be mediated by specific proteins.

A genome wide survey of putative Arabidopsis plastid inner membrane proteins through bioinformatics approaches is presented in chapter 4. Among the candidates identified were ATP binding cassette transporters, many of which have been implicated as lipid transporters in other systems (see review below). Initial efforts toward identification of potential plastid envelope lipid transporters through reverse genetics approach are described within the chapter.

Fatty acid derived storage and structural lipid syntheses

In seeds, fatty acids are precursors of triacylglycerol (TAG) biosynthesis, the major sink of carbon in oilseeds. Some of the steps involved in triacylglycerol synthesis and many aspects of subcellular compartmentation are the same as lipid synthesis in leaves (Stymne and Stobart, 1987; Browse and Somerville, 1991; Miquel and Browse ,1994). As with the eukaryotic pathway, PA for TAG synthesis is made in the ER and the dephosphorylation of resulting PA forms DAG. In a TAG synthetic pathway called Kennedy pathway, a third FA is transferred to the sn-3 position of DAG, catalyzed by diacylglycerol acyltransferase (DAGAT) (Ohlrogge and Browse, 1995). Alternatively DAG can be formed from PC. PC participates in TAG formation by either donating it's entire DAG moiety (Slack et al., 1983) or providing acyl moieties to the acyl-CoA pool (Stymne and Stobart, 1987). TAG can also be synthesized in an acyl-CoA-independent

mechanism, which uses phospholipids as acyl donors and DAG as acceptor catalyzed by phospholipid-diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000).

Relatively less is known about the syntheses of structural lipids such as wax, cutin and suberin. The aliphatic components of the cuticular wax are synthesized in the epidermal cells from the saturated very long-chain fatty acids (VLCFA, 20-34 carbons). The C16 and C18 FA's synthesized in the plastid are elongated into the VLCFA by extra-plastidial membrane-associated multienzyme complexes, known as the fatty acid elongases (FAE) (Fehling and Mukherjee, 1991; Kunst and Samuels, 2003). The VLCFA's are further modified to produce the primary alcohols, aldehydes, alkanes, secondary alcohols, and ketones, which contribute to the wax formation (Cheesbrough and Kolattukudy, 1984). The principal constituents of cutins and suberins are also derived from the saturated and unsaturated FA's that are hydroxylated, epoxidated, and crosslinked to form a network (Kolattukudy, 2001).

Problem

Free fatty acids are toxic to the cell and could cause deleterious effects on membrane integrity. Therefore the cell must control the accumulation of free fatty acids during the synthesis and degradation of storage and structural lipids. During the germination of seeds, TAG stored in the oil body needs to be immobilized to be used to provide carbon and chemical energy. Catabolism of fatty acids occurs exclusively in the glyoxysome in plants. Therefore, once the fatty acid gets liberated from the glycerol backbone of the TAG, they should be funneled into the glyoxysome to be oxidized. Other than the

microscopic observation that during germination, the oil body and glyoxysome come in close physical contact which is hypothesized to facilitate the transfer of fatty acids, not much is known about this process (Mollenhauer et al., 1970; Vigil et al., 1970; Wanner et al., 1982). As will be reviewed later, a membrane transporter is thought to be involved in the internalization of fatty acids into the glyoxysome. How the hydrophobic building blocks of structural lipids are moved from the ER to the site of polymerization and eventually out of the plasma membrane is also currently not known (Kunst and Samuels, 2003).

A set of experiments dealing with exogenously provided fatty acid metabolism within the cell are presented in Chapter 3. Plant cells possess a system to accommodate excessive amounts of free fatty acids by incorporating them into their endogenous lipids. A stromal localized protein is involved in the activation of medium chain fatty acids for integration into the regular plastid FAS machinery.

Membrane Transport of Free Fatty Acids: The Debate over Passive Diffusion versus Facilitated Transport

The studies of membrane transport of unesterified FA's reviewed in this section are mostly adapted from animal system where release and uptake of FFA's to/from plasma by different types of cells is a routine process. Although plant cells are conceptually different in respect to exchanging FFA between cells, they require massive FFA exchange between subcellular compartments as reviewed above. FA movement across

biological membrane is essential to the physiology of all living organisms and thus may have conserved aspects across the bacterial, plant and animal kingdoms.

Dietary triglycerides digested by gastric and pancreatic enzymes are emulsified with bile salts in the digestive tract and are internalized by intestinal epithelial cells (Frohnert and Bernlohr, 2000). Free fatty acids are released from circulating TAG's by endothelial lipoprotein lipases for uptake by multiple tissues including adipose, skeletal and heart muscles. In the resting state, FA's are the heart's primary fuel source. Fuel reserves in the plasma are essentially negligible and well-oxygenated heart muscle cells receive FA via the plasma from adipocytes in distal sites (Neely and Morgan, 1974). Adipose TAG's are the single largest energy depot, enough to maintain life for about three months (Cahill Jr., 1970). FA must leave the adipocytes, cross the capillary endothelium, travel through the blood stream, and eventually enter the cytosol of the heart cell (Hamilton, 1998).

The amphipathic nature of FFA's has given rise to a major and long-standing debate over whether they move across cell membranes by passive diffusion, or by facilitated transport mechanism. Some investigators have proposed that the passive diffusion of FA is slow and thus is the rate-limiting step for FA transport in model membranes (Kleinfeld et al., 1995; Kleinfeld, 2000). In direct contrast others argue that simple diffusion by lipid physical chemistry is fast and sufficient to match the physiological requirements (Kamp et al., 1995; Hamilton, 1998; Hamilton and Kamp, 1999; Kamp et al., 2003). Some investigators have suggested transporter involvement under low concentrations of FFA and passive diffusion at higher levels of FFA (Abumrad et al., 1981) or vise versa;

passive diffusion under basal condition and transporters that increase the uptake above the basal level (Schaffer and Lodish, 1994).

Diffusional model

The physical chemistry studies of FA transport involve use of model membrane vesicles. Model vesicles are mostly a single lipid bilayer encapsulating aqueous buffer. Small unilamellar vesicles (SUV's) have around 250 Å diameter and are prepared by sonication. The large unilamellar vesicles (LUV's) have a lower surface curvature and have a higher variability in diameter (usually < 500 nm). LUV's can entrap smaller vesicles. The membrane may be made with phospholipids of choice with or without proteins (Hamilton, 1998). In classical uptake studies, membrane vesicles are incubated with FA's in a solution containing albumin and initial uptake rates are evaluated by measuring radiolabled FA's bound to or trapped in the vesicles (Hamilton and Kamp, 1999; Stump et al., 2001). Alternatively, ^{13}C NMR spectroscopy was used to monitor the FA binding to albumin or to phospholipids by characteristic chemical shifts of the carboxyl carbon (Hamilton and Cistola, 1986). Fluorescence was also used to monitor FA transfer from a donor to an acceptor (Noy and Zakim, 1985; Kamp and Hamilton, 1993). The transbilayer movement of FA was monitored by detecting the change in pH inside the vesicles using a fluorescent pH probe (pyranin) (Kamp and Hamilton, 1992; Kamp et al., 1993).

The diffusional model views passive diffusion of FA through the lipid bilayer as the central mechanism of transport of FA into and out of cells and predicts that in general FA metabolism regulates transport of FA rather than the reverse (Hamilton, 1998). Three minimal steps are involved in membrane FA transport by passive diffusion model; 1) adsorption from extracellular media (albumin) to the lipid bilayer, 2) transmembrane flip-flop, 3) desorption from lipid bilayer to the cytosol.

Various studies have demonstrated the preferential partitioning of long-chain FA's into membranes as opposed to water. The partition coefficient ($[oleate]_{membrane}/[oleate]_{water}$) of oleic acid in model phospholipids vesicles was 0.5×10^6 (Richieri et al., 1995; Kamp et al., 2003). The calculated rate constant ($K_{eq} = k_{off}/k_{on}$) of palmitate to dimyristoyl PC SUV was 2.4×10^7 s^{-1} (Hamilton, 1998). If albumin is a donor the partitioning of FA between albumin and the membrane will be affected by factors such as the FA to albumin ratio, the type of FA, the relative amounts of membrane and albumin, the membrane composition, the pH, and temperature. ^{13}C-NMR spectroscopy demonstrated a reversible transfer of albumin bound oleate to phospholipid bilayers with exchange times in the range of $0.1\ s < t_{1/2} < min$ (Hamilton and Cistola, 1986).

Membrane permeability coefficients for short- and medium-chain FA's (up to 12 carbons) are high and increases with chain length (Antonenko et al., 1993; Zakim, 1996).

The un-ionized FA move across the bilayer rapidly ($t_{1/2} < 1$ s, Kamp and Hamilton, 1992) compared to the anion. However, if FA is present in a memberane exclusively as anions, FA diffusion across membrane must be slow and will have to depend on anion transporters. Several investigators have shown that the apparent pK of the FA in SUV is about 7.5, close to physiological pH (Ptak et al., 1980; Hamilton and Cistola, 1986). About 50% of the FA population in the phospholipid bilyer is expected to be in un-ionized form in the physiological pH range. Thus it is possible that FA could diffuse across the lipid bilayer via ther unionized form (Hamilton, 1998). There seems to be disagreement between the measurements of LCFA's in larger size model vesicles. Keinfeld et al. (1998) reported an order of magnitude decrease in the rate of flip-flop in PC/cholesterol vesicles when the diameter was raised from 100 nm (LUV's) to 200 nm (giant unilamellar vesicles). In pancreatic β-cells, the rate of acidification upon addition of LCFA was slower than for LUV's by several orders of magnitude (Hamilton et al., 1994). Kamp et al. (2003) used both vesicles of the plasma membrane (protein/phospholipids ratio, 1:1 (w/w), 100-500nm diameter) and intact adipocytes. Their reported partition coefficients of 1×10^6 for plasma membrane vesicle and 1.4×10^6 for adipocytes were similar to that of protein free model membranes. Oleic acid flip-flop across the plasma membrane vesicle and adipocyte cell was measured by the change in fluorescent pH probes in the vesicle and was fast ($t_{1/2} < 5$ s). Both in LUV's and in cells, the lack of dependence of the rate of pH change on FA chain length or structure (FA dimmers or alkylamines) suggested a non-protein mediated mechanism.

Desorption of FA from a phospholipid surface is slower than flip-flop and dependent on the FA chain length and the level of unsaturation. The spontaneous desorption of typical FA from a PL interface when evaluated from SUV to acceptor SUV was reported to be fast ($t_{1/2}$ < 1 s) but was slower for very long-chain FA's ($t_{1/2}$ about 30 min for C24) (Massey, 1997; Zang et al., 1996). These studies support the conclusion that the physical properties of FA movements across the membranes are rapid and do not require protein-mediated transport.

However, there are several caveats to the above arguments that may weaken such a simple conclusion. First, the studies that show fast movement of FA either in model vesicles or cells do not rule out the possibility that alternate or additional mechanisms exist particularly in cells with high FA uptake (Kleinfeld et al., 1997). Second, the spontaneous movement of FA's in these model membrane vesicles may not necessarily be reflected in biological system where extracellular components such as the collagen matrix, non-lipid components in the membrane and the unstirred water layer affects the physical properties of FA interaction with membranes (Richieri et al., 1995; Kleinfeld, 2000). Third, the internal acidification upon FA entry into the vesicle which was used to probe the FA flip-flop may be attributed to an indirect effect in cases with cell metabolism (Abumrad et al., 1998). The volume in LUV's is 10^4 times less than that of an average cell (20μm) and the changes in pH measured in larger volume compartments will be smaller. The flip-flop of FA in biological membranes may be much slower, where the different curvature, lipid composition, and the presence of membrane proteins might slow down FA diffusion (Abumrad et al., 1998; Berk and Stump., 1999; Schaffer

et al., 2002). Fourth, it turns out that the ratio between albumin and FA, especially the unbound FA concentration, is a very important factor in determining kinetics. Many of the physical chemistry experiments to measure FA diffusion into the membrane vesicles were carried out at very high FA to albumin ratios (Abumrad et al., 1998). Fifth, the relative contribution of the protonated and ionized forms to FA uptake remain uncertain. Sixth, although there is agreement that short-chain FA can diffuse very quickly across the membrane, reported measurements of long-chain FA's differ considerably (Kleinfeld and Storch, 1993; Kleinfeld et al., 1997). Seventh, flip-flop of nonionized FA's within the membrane and subsequent ionization are not necessarily equivalent to *in vivo* transport of FA for metabolism (Schaffer, 2002).

Facilitated transport model: kinetic evidence for protein-mediated transport

One of the key arguments for facilitated FA transport came from the saturation kinetics observed for FA uptake into isolated rat adipocytes (Abumrad et al., 1981, 1984). The uptake of FA tracers was carried out at physiologic albumin concentration. At each time point, uptake was stopped by addition of ice-cold solutions containing 0.1% BSA and phloretin. Phloretin inhibits further influx or efflux of FA whereas BSA removes any FA's that adhere to the cell surface. When FA and albumin are both present in the extracelluar medium, the kinetics of uptake follows the molar ratio of FA to albumin rather than the total FA concentration (Sorrentino et al., 1989; Spector et al., 1965). Initial rates of LCFA transport into isolated adipocytes were a function of unbound FA concentration in the medium, and demonstrated to have both saturable and nonsaturable

components. The saturable components are observed at low (< 3.0) LCFA to BSA ratios while the kinetics exhibits nonsaturable components with the increase of the ratio. The ratio in normal human serum is about 0.74 (Richieri and Keinfeld, 1995). The measured K_m from transport was 7nM for oleate which is in the range of circulating concentrations of unbound FA's (Abumrad et al., 1998). The saturable component is specific for LCFA's but not for medium-chain fatty acids (MCFA's). Saturation characteristics were also observed later with hepatocytes, cardiac myocytes (Stremmel and Berk, 1986), Caco2 human intestinal cells (Kim and Storch, 1992), myocytes (Stremmel, 1988; Luiken et al., 1997), and giant sarcolemmal membrane vesicles from rat hindlim skeletal muscles (Bonen, 1998).

A second argument comes from competition experiments where competition for uptake was observed between different substrates. Competition was demonstrated among long-chain FA's but not short-chain FA's with different cells including adipocytes (Abumrad et al., 1984; Storch et al., 1991), hepatocytes (Sorrentino et al., 1996), and an intestinal cell line (Trotter et al., 1996). In a study with a cultured cell line of adipocytes, a fluorescent FA analogue that can not be metabolized in the cell was used as competitor (in order to avoid the possibility of different demand of FA's in cell metabolism being the cause of observed competition) but competition was still observed (Storch et al., 1991).

The third evidence for protein mediated transport is derived from studies that used protein modification reagents to block the FA transfer process. Transport was inhibited by phloretin, pentachlorophenol, diisosalicylate, quercetin, diisothiodisulfonic acid,

sulfosuccinimidyl derivatives of oleate and myristate, photoaffinity labeling reagent 11-azistearate derivative and in some cases with proteases such as pronase and trypsin in a variety of cells tested including hepatocytes, cardiac myocytes, Caco2 intestinal cells, myocytes, giant sarcolemmal membrane vesicles, rat renal basolateral membrane vesicles (Abumrad et al., 1998; Frohnert and Bernlohr, 2000). Many of the reagents were known anion transport inhibitors which may suggest that the transported fraction of FA's is in ionized form.

It is possible that both diffusional and facilitated mechanisms are operative in the movement of FA's across the membranes with the relative contribution being different at different physiological conditions, with different FA needs for different metabolic processes, or with different FA substrates. Studies of hepatocytes and adipocytes, conducted over a wide range of FA to BSA ratio specifically identified both saturable and nonsaturable uptake components (Stump et al., 1992; Stump et al., 2001). At the ratio ranging from 0.5 to 3, more than 90% of uptake was via the saturable pathway. As the ratio increased, the linear process took over. Within the physiological range of 0.5-3, rate constants for saturable transmembrane influx were 2.9 s^{-1} and were 10 to 30 fold faster than those for linear uptake ($k = 0.1 - 0.26\ s^{-1}$, $t_{1/2} = 2.7 - 6.6$) (Stump et al., 2001). Cell-specific changes in the Vmax for saturable FA uptake occur in human disease states including obesity, type 2 diabetes, alcoholic liver disease, and cardiomyopathy (Zhou et al., 1992; Berk et al., 1997, 1999; Stump et al., 2001). In addition a syndrome of recurrent episodes of severe liver failure in children was attributed to a defect in LCFA membrane transport (Odaib et al., 1998).

Some of the counter arguments to the facilitated model include the following. First the observation of saturation kinetics is not sufficient evidence for protein transport and may reflect partitioning of FA between membrane and albumin (Kamp et al., 1993). Secondly, reagents used to modify proteins to show inhibition of FA uptake may interfere with intracellular lipid metabolism or may alter the membrane structures that resulted in the physical partitioning of FA (Kamp et al., 2003). Thirdly, most of the studies supporting protein-mediated transport were conducted with the transmembrane step not being isolated from adsorption and desorption steps. Fourth, although FA uptake was affected by expression or suppression of FA transport related proteins, the actual mechanism of action has not been established and thus it is hard to know whether they are directly involved in transport *per se* or are involved in the overall uptake process indirectly (Hamilton, 1998).

Candidates of Lipid Transporters Identified in Various Organisms

Proteins that are thought to be involved in the various aspects of lipid movement have been accumulated over many years in different organisms using different experimental strategies. Some candidate proteins are supported with more convincing functional data while others lack evidence. Thus the level of confidence may vary between the candidates. In order to assist better judgment on such candidates short details are provided in this section. Table 2 summarizes the candidates reviewed in this section.

They are organized into 4 main groups; 1) unesterified FA transporters, 2) activated FA transports, 3) membrane lipid transporters, and 4) plant lipid transporters.

Unesterified FA transporters

***Escherichia coli* FadL and FadD**

E. coli can be grown under condition where *de novo* LCFA biosynthesis is blocked by the FAS inhibitor cerulenin and providing LCFA's in the media as the sole carbon source. This feature makes genetic screening possible for mutants that can not grow under this condition. The outer membrane of *E .coli* is composed of an external layer of lipopolysaccharide and an internal layer of phospholipids associated with a layer of peptidoglycan. The lipopolysaccharide is refractory towards hydrophobic compounds such as LCFA's. The outer membrane and inner membrane are separated by a periplasmic space. Transport of LCFA across these barriers is necessary under the above screening condition. The screen led to identification and mapping of the fadL gene, encoding the long-chain fatty acid transport protein FadL (Nunn and Simons, 1978). The FadL protein has binding specificity toward long-chain fatty acids, and the carboxylate of the fatty acid is essential for ligand binding (Black, 1990; Black and Zhang, 1995). FadL spans the outer membrane and is composed of two discrete domains; the amino terminal domain is exposed at the surface making it protease sensitive and is involved in ligand binding, and the carboxyl terminal domain is embedded within the membrane and is thought to form LCFA specific channel. Two allelic mutations in the transmembrane region keep FadL in an open conformation (Kumar and Black, 1991, 1993).

Table 2. Summary of identified lipid transporter candidates.

Classification	Name	Organism/ organelle	Example match in Arabidopsis [1]
Unesterified FA transporters	FadL, FadD, Tsp, H^+/FA cotransporter	bacteria	5 genes for Tsp (E < 9e-7) [2], AMP binding protein family for FadD
	Fat1p, Faa1p, Faa4p	fungi	AMP binding protein family
	FABPpm	animal	7 genes (E < e-115)
	FAT/CD36	animal	No significant [3]
	FATP family	animal	AMP binding protein family
	Caveolin, 56 kDa renal FA binding protein	animal	No significant
Activated FA transporters	Carnitine system; CPT-I, CACT, CPT-II	mitochondria	mitochondrial substrate carrier family for CACT
	long-chain acyl-CoA transporters; Pat1p, Pat2p, ALDp	peroxisome	PXA1, ABC transporter family
Membrane lipid transporters	Scramblase	animal	No significant
	Aminophospholipid translocase; Rh protein, PS-stimulated Mg2+ ATPase, chromaffin granule ATPase II	animal	ALA1, haloacid dehalogenase-like hydrolase family
	Multidrug Resistance Proteins; Mdr2, MDR1, MDR3, MRP1, ABCR, ABC1, MsbA	animal, bacteria	ABC transporter family
	Interorganellor contact zones (MAM); MET30	fungi	WD-40 repeat family
Plant lipid transporters	PXA1, VIPP1, TGD1, ALA1	plant	N/A

[1] Blast search was performed using 'The Arabidopsis Information Resource (TAIR) blast' program (URL http://www.arabidopsis.org/Blast/) using peptide sequences.

[2] The 'Expect value (E)' is from the TAIR blast and is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.

[3] E > 0.01 is considered insignificant in this table but no 'defined' E value for insignificant homology exists.

The LCFA transport is shown to be shock-sensitive, which supports the existence of a periplasmic component, although no specific periplasmic FA binding proteins were identified. The periplasmic protease Tsp is required for optimal levels of transport and is thus believed to be peripherally involved (Azizan and Black, 1994). An oleate binding protein is postulated to be an inner membrane H^+/FA cotransporter but remains to be proved (Kameda et al., 1987).

Another gene that was identified from the genetic screening is fadD (Black et al., 1992). FadD belongs to the family of long-chain fatty acyl-CoA synthetases which catalyzes the formation of fatty acyl-CoA from FFA. FadD is routinely isolated from the cytosolic fraction although there is evidence that it becomes membrane-bound in response to specific physiological conditions (Mangroo and Gerber, 1993).

Yeast Fat1p and Faa1p, Faa4p

The LCFA uptake by yeast follows saturable kinetics suggesting involvement of facilitated transport (Kohlwein and Paltauf, 1983). Disruption of the *FAT1* gene in yeast resulted in impaired uptake and growth on LCFA (Faergeman et al., 1997). Deletion mutants can not grow on media containing cerulenin and LCFA. Furthermore the mutants are impaired in accumulation of the fluorescent FA analogue BODIPY-3823, myristate, palmitate and oleate. Fat1p belongs to the family of adenylate forming enzymes which contain ATP/AMP binding domain but lacks the long-chain fatty acyl-CoA synthetase signature (Faergeman et al., 1997). Fat1p has high similarity to the murine fatty acid transport protein FATP identified from 3T3-L1 adipocytes (Schaffer

and Lodish, 1994). Fat1p is predicted to span the membrane four times, with the ATP/AMP binding site residing on the cytoplasmic side. Upon entry the LCFA's are activated to CoA thioesters by Faa1p and Faa4p (Johnson et al., 1994). Strains carrying deletions in *FAA1* and *FAA4* are phenotypically similar to the *FAT1* deletion mutants. It has been hypothesized that there is a mechanistic linkage between the import and activation of LCFA, and that one or both of Faa1p or Faa4p are the components of fatty acid transport system in yeast (Faergeman et al., 2001).

Mammalian FABPpm

FABPpm was purified from solubilized rat liver plasma membranes and jejunal microvillous membranes by oleate-agarose affinity chromatography (Stremmel et al., 1986). FABPpm is expressed on the plasma membrane of liver, adipose tissue, cardiac muscle, intestine, vascular endothelium and internal membranes where FA uptake/export is highly active (Schwieterman et al., 1988; Sorrentino et al., 1988). FABPpm and mitochondrial aspartate aminotransferase (mAspAT) are shown to be identical proteins (Stump et al., 1993). When mAspAT was expressed on the surface of fibroblasts the oleate uptake was increased (Isola et al., 1993) and antibodies to mAspAT/FABPpm reduced FA uptake in various cells (Stremmel and Theilmann, 1986; Zhou et al., 1992,1995). Consistent with the postulated function of fatty acid uptake, FABPpm expression was found to be upregulated during adipocyte differentiation (Dutta-Roy et al., 1996). The FABPpm expression was also found to change in adipocytes of diabetic rats (Berk et al., 1997), during endurance training (Kiens et al., 1997) and fasting

(Turcotte et al., 1997). The mechanism by which this globular protein is targeted and tethered to the plasma membrane is unclear.

Mammalian FAT/CD36

Fatty acid translocase (FAT, 88kDa) was identified by covalent labeling with radiolabeled inhibitors of the FA transport step such as 4,4'-diisothiocyanostilbene-2,2' disulfonate (DIDS), sulfosuccinimidyl myristate (SSM) or sulfo-N-succinimidyl oleate (SSO) from adipocytes (Harmon and Abumrad, 1993). FAT has sequence similarity to the human platelet integral membrane glycoprotein CD36 that functions as a multiligand scavenger receptor (Abumrad et al., 1993). CD36 is found in tissues with a high metabolic capacity for FA. Expression of CD36 in fibroblasts which normally lack this protein induces appearance of a saturable, high affinity, phloretin-sensitive component of FA uptake (Ibrahimi et al., 1996). CD36 expression is also increased in the muscle of diabetic animals, in mice fed with a high fat diet, and is regulated by change in FA utilization (Abumrad et al., 1998). CD36 overexpressing mice have decreased blood levels of TAG and FA's (Stremmel, 1988), whereas CD36 knockout mice have increased fasting levels of FFA and TAG in serum and reduced uptake of FFA in isolated adipocytes (Ailhaud, 1993). In humans, CD36 deficiency has been reported to be linked with heart muscle hypertrophy or dysfunction (Shaffer, 2001).

FATP family conserved from mycobacterium to mammals

A member of the FATP family called *mus musculus* fatty acid transport protein 1 (mmFATP1) was first identified using a technique called expression cloning (Schaffer

and Lodish, 1994). An adipocyte cDNA library was expressed in COS7 cells and the cells were screened for the internalization of a fluorescent fatty acid analog. FATP1 is a 646 amino acid integral plasma membrane protein with a long hydrophobic amino-terminal region that faces the extracellular space. FATP1 has two domains that have sequence similarities to long-chain acyl-CoA synthetases and very long-chain acyl-CoA synthetase (Uchiyama et al., 1996; Schaffer, 2001). Hirsch et al. (1998) reported the presence of 4 other murine homologues and the human homologues of all 5 members plus a sixth member. FATP homologues were also present in C. *elegans*, *M. tuberculosis* and *Drosophila melanogaster* (Hirsch et al., 1998).

Transfection experiments of mmFATP1, mmFATP2, mmFATP5 into 3T3 fibroblasts and COS cells, resulted in increased FA and FA analogue uptake in the transfected cells (Schaffer and Lodish, 1994; Hirsch et al., 1996). Non-mammalian homologues also exhibited FA transport activity when transfected to COS cells and *E. coli* (Schaffer, 2001). The expression of FATP1 increases during adipose development (Man et al., 1996). A Human homologue of FATP4 (hsFATP4) shows polarized distribution in enterocytes, with highest levels on the luminal side of the cell, indicating a possible role in dietary FA uptake from intestinal lumen. Antisense depletion of FATP4 significantly reduces LCFA import in murine enterocytes (Stahl et al., 1999). Insulin down regulated whereas fasting upregulated mmFATP1 mRNA expression in mouse adipose tissue (Man et al., 1996).

Other mammalian FA binding proteins

Caveolin, a 22kDa integral membrane protein, was identified by using a photoreactive FA analogue from 3T3-L1 preadipocytes (Trigatti et al., 1991, 1993). Caveolin showed high affinity for FA's, and mRNA expression was increased during adipocyte differentiation. Later it was reported to be a structural component of caveolae, the flask-shaped invaginations of the cell membrane which play a role in endo- and exocytosis (Mangroo et al., 1995; Trigatii et al., 1999). The functional aspect of caveolin in FA transport is not clearly distinguished from its role in endocytosis (Frohner and Bernlohr, 2000).

The 56 kDa renal FA binding protein was purified to homogeneity from rat renal basolateral and cardiac myocyte membranes by a series of purification steps including oleate-Sepharose 4B affinity chromatography (Fujii et al., 1987). However, no follow-up experiments showing its role in FA transport have been reported (Hui and Bernlohr, 1997).

Activated FA transporters

Mitochondrial carnitine system

FA oxidation in animal cells takes place in peroxisomes and in mitochondria matrix. The FA entry across the double membrane of mitochondria is achieved by the well-studied carnitine system. The mitochondrial carnitine system consists of at least 3 enzyems; carnitine palmitoyltransferase I (CPT-I), carnitine:acyl carnitine translocase (CACT) and carnitine palmitoyltransferase II (CPT-II) (Kerner and Hoppel, 2000). FA's from cytosol

are first activated to acyl-CoA by a long-chain acyl-CoA synthetase located on the mitochondria outer membrane (Kerner and Hoppel, 2000). Long-chain acyl groups are transferred to free carnitine to form long-chain acyl-carnitine, catalyzed by the outer membrane-localized CPT-I (McGarry and Brown, 1997). Long-chain acyl-carnitines are then translocated into the mitochondrial matrix in an exchange reaction catalyzed by an integral inner membrane protein, CACT (Indiveri et al., 1990, 1995). Within the matrix the long-chain acyl-carnitines are reconverted to long-chain acyl-CoA by CPT-II. This enzyme is anchored to the inner membrane as a peripheral membrane protein (Bieber, 1988).

CPT-I represents a key regulatory site controlling the flux through β-oxidation. CPT-I is inhibited by malonyl-CoA but under starvation and diabetic conditions becomes less sensitive to malonyl-CoA, allowing maximal FA oxidation (McGarry and Foster, 1979; Drynan et al., 1996). CPT-I shows developmental stage-specific expression and is subject to transcriptional controls by exogenous FA treatments, fasting, insulin. The posttranslational modification-dependent interaction with cytoskeletal components are also implicated in the regulation of CPT-I activity (Thumelin et al., 1994; Park et al., 1995; Chatelain et al., 1996; Guzman et al., 1994; Velasco et al., 1998).

Peroxisomal LCFA membrane transporters

In yeast, FA oxidation takes place exclusively in peroxisomes (Hettema and Tabak, 2000). The LCFA's are activated to the long-chain acyl-CoA on the cytosolic side of the peroxisome membrane (Johnson et al., 1994; Hettema et al., 1996). Activated long-chain

fatty acyl-CoA's are transported across the peroxisomal membrane by peroxisomal ABC half transporters, Pat1p (also known as Pxa2p) and Pat2p (also know as Pxa1p). Pat1p and Pat2p are shown to form a heteromeric complex (Shani and Valle, 1996). Pat1p and Pat2p are induced upon growth on oleate and are necessary for normal growth on LCFA's as a sole carbon source (Hettema et al., 1996; Shani and Valle, 1996). The *PAT1/PAT2* deletion mutants have considerably reduced β-oxidation activity.

Four different peroxisomal ABC half-transporters have been identified in mammals and some of them are shown to be partially redundant (Braiterman et al., 1998). X-Linked adrenoleukodystrophy patients accumulate VLCFA's due to a defect in a peroxisomal ABC transporter (ALDp), and it is likely that this transporter is involved in transport of VLCFA's across the peroxisomal membrane (Mosser et al., 1993, 1994).

Membrane lipid transporters

The asymmetric distribution of lipids across the bilayer is a fundamental property of most biological membranes (Bretscher, 1973; Rothman and Lenard, 1977; Op den Kamp, 1979). Disruption of the asymmetry is part of the normal blood clotting mechanism but in other cases results in defects in signal transduction pathways, defects in macrophage recognition of senescing cells, and change in activities of cytoplasmic proteins. A number of human diseases including Scott syndrome (Bevers et al., 1992), sickle cell disease (Lubin et al., 1981), stroke (Ciavatti et al., 1978) and diabetes (Wali et al., 1988) are related to problems in maintaining the homeostasis of this asymmetry.

Transbilayer lipid asymmetry is established during biosynthesis of each class of lipid (van Meer, 1998). Once asymmetry is generated, spontaneous transbilayer lipid flip-flop is slow ($t_{1/2}$= hours to days) and is inversely proportional to fatty acyl chain length and hydrophobicity (Kornberg and McConnell, 1971; Middelkoop et al., 1986). Protein-lipid interactions partly help in preventing certain lipids from traversing to the other side of the bilayer thus contribute to the maintenance of the asymmetry (Cohen et al., 1988), but a specialized set of transporters are reported (as reviewed below) to play important roles in the transbilayer movement of membranelipids.

Human erythrocyte scramblase

The scramblase plays a key role in the stimulation of plasma clotting factors by platelets and in the recognition of senescent and apoptotic cells by macrophages (Bevers et al., 1982; Conner et al., 1994). Human erythrocyte and platelet scramblases were purified and subsequently cloned (Bassé et al., 1996; Comfurius et al., 1996; Zhou et al., 1997). This protein catalyzes the externalization of PS in response to rising Ca^{2+} concentrations during cell activation which results in the lipid-randomizations. This protein is an integral membrane protein with potential palmitoylation sites and a proline-rich cytoplasmic N-terminus (Zhao et al., 1998; Zhou et al., 1998). The size (35kDa) and predicted membrane topology lead to a speculation that this protein is not a monomeric transporter but may form either a functional oligomer or act as a subunit of a larger complex (Daleke and Lyles, 2000).

Aminophospholipid translocase

Energy-dependent, cytofacially directed PS and PE transport activity was reported and confirmed in human erythrocytes (Seigneuret and Devaux, 1984; Loh and Huestis, 1993). This activity requires ATP and is sensitive to Ca^{2+}, vanadate, N-ethylmaleimide, and exhibits specificity for PS. Some purified proteins, including human erythrocyte Rh protein, human erythrocyte PS-stimulated Mg^{2+} ATPase, and chromaffin granule ATPase II fit into this category and are thus candidates of aminophopholipid translocase.

The Rh protein was found by its selective reactivity with a radiolabeled sulfhydryl reagent and with PS photoaffinity probe (Connor and Schroit, 1988). However, direct evidence is still lacking that this protein is indeed an aminophospholipid translocase (Schroit and Zwaal, 1991). The PS-stimulated Mg^{2+}-ATPase was partially purified from human erythrocyte following the biochemical properties of aminophospholipid translocase, namely, activation by PS, and the Mg^{2+}-ATPase activity (Morrot et al., 1990; Zimmerman and Daleke, 1993; Auland et al., 1994). The partially purified samples were able to catalyze ATP-dependent movement of PS and PE in the reconstitution experiments (Auland et al., 1994). The bovine chromaffin granule ATPaseII is functionally similar to the erythrocyte PS-stimulated Mg^{2+}-ATPase, and was purified and cloned (Moriyama and Nelson, 1988; Tang et al., 1996). This protein contains P-type ATPase consensus sequences with 10 transmembrane spanning domains. Drs2p from yeast when mutated shows aminophospholipid transport defect phenotype and has

sequence similarity to ATPaseII suggesting the possible role of ATPaseII in the aminophospholipid transport (Tang et al., 1996).

Multidrug Resistance Proteins

The phosphatidylcholine (PC) translocator was first found in mouse null mutant of Mdr2 gene. The homozygous knockout mouse can not secrete PC into bile, and the absence of PC in bile resulted in mild liver disease in mice. Deficiency in human Mdr2 homologue MDR3 (sometimes called MDR2), leads to a serious liver disease requiring transplantation (Smit et al., 1993; Schinkel et al., 1997). Both Mdr2 of mouse and human MDR3 encode P-glycoproteins (Pgp) which belong to the ATP binding cassette (ABC) transporter family. The direct evidence of Mdr2 involvement in PC transport was demonstrated in a yeast mutant transfected with an Mdr2 cDNA construct (Ruetz and Gros, 1994). The Mdr2 protein was able to catalyze the direct translocation of a PC analogue and displayed high specificities for phospholipid analogues with a PC head group. MDR1 is also able to translocate lipid analogues. However, they show specificity toward a wide range of molecules and their function as lipid transporters is still unclear.

Another set of ABC transporters known as multidrug resistance associated proteins (MRP's) are also able to transport lipid analogues (Cole et al., 1992; Kool et al., 1997). MRP1 is an organic anion transporter with high activity toward glutathione conjugated compounds. The physiological evidence of MRP1 involvement in lipid metabolism is still lacking (van Helvoort et al., 1996).

ABCR (or Rim protein), a distant relative of Pgp, was found to be defective in age-related macular degeneration called Stargardt's macular dystrophy (Allikmets et al., 1997). ABCR appears to function as an outwardly-directed flippase for N-retinylidene-PE.

ABC1, another distant relative of Pgps, is linked with atherosclerosis (Young and Fielding, 1999). This protein is one of the largest ABC transporters known with 2201 amino acids. When mutated it results in familial high density lipoprotein deficiency and when is completely absent rapid degradation of high density lipoproteins in plasma membrane leads to a disease known as Tangier disease (Rust et al., 1999). Further research indicated that ABC1 is responsible for cholesterol efflux from cells (Borst et al., 2000).

MsbA is a bacterial Mdr homolog (Karow and Georgopoulous, 1993). Lipid A, a hexa-acylated disaccharide of glucosamine, is the hydrophobic anchor of lipopolysaccharide that covers the outer membrane of Gram-negative bacteria. The enzymes that make lipid A are located in the cytoplasm or inner membrane. MsbA is demonstrated to be an essential component of lipid A transport across the inner membrane and possibly to the outer membrane (Polissi and Georgopoulos, 1996; Zhou et al., 1998; Doerrler et al., 2001).

Interorganellor phospholipids transport via contact zone

PS can serve as a precursor for PE and PC synthesis in eukaryotes. PS is synthesized in the ER but PS decarboxylase is found in mitochondria and Golgi-vacuole (Voelker, 1989). PE synthesized by PS decarboxylase then returns to ER where methylation occurs to form PC. A specialized zone of ER called mitochondria-association membrane (MAM) was implied to be involved in these exchanges of phospholipids (Vance, 1990; Gaigg et al., 1995). Close association between Golgi and ER, and ER and plasma membrane was also observed (March et al., 2001; Pichler et al., 2001). The MAM fraction is found to be enriched in PS synthase. Direct evidence that MAM or other contact zones are involved in interogranllor transfer of lipids is still lacking (Voelker, 2003). Genetic analyses in yeast lead to the finding of mutants (*pstA1*) defective in mitochondrial PS transport (Schumacher et al., 2002). The *MET30* gene complemented the deficiency in the mutant. *MET30* encodes a subunit of a multicomponent ubiquitin ligase. There are increasing evidences of ubiquitination role in membrane trafficking regulation (Hicke, 2001).

Lipid transporters in plants

There is almost no physical biochemistry work documented in plants concerning unesterified FA transport. However, some proteins that are implicated to be involved in several aspects of lipid transport in plant cells are beginning to be discovered, mostly through genetic analyses.

PXA1 (also discovered independently and named *PED3* or *CTS* by separate groups) is an Arabidopsis version of the yeast peroxisomal long-chain acyl-CoA transporters Pat1p and Pat2p, as well as the human peroxisomal ABC transporter (ALDP) (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002). The *pxa1* mutant displays β-oxidation defect phenotypes. In addition this mutant was found to accumulate very long-chain acyl-CoA's during the germination on sucrose supplied media. This and other data suggested that the VLCFA's are transported into the peroxisomes as activated acyl-CoA, consistent with the observations made in yeast and mammals (Hettema et al., 1996; Mannaerts et al., 1982).

Thylakoid membrane components are synthesized in the inner and outer membrane of chloroplasts. Vesiculation of the inner membrane seems to be the carrier of membrane elements for thylakoid membrane biogenesis and maintenance (Morré et al., 1991; Kroll et al., 2001). A pea 37 kDa protein, IM30, was found both in the chloroplast envelope and in the thylakoid, and was hypothesized to be a candidate for the transfer of galactolipids from the envelope to thylakoid (Li et al., 1994). Lack of vesicle formation was paralleled by the inhibition of thylakoid formation in an Arabidopsis mutant disrupted in the Arabidopsis homolog of IM30, the vesicle-inducing proteins in plastids 1 (*VIPP1*) (Kroll et al., 2001). Deletion of the *VIPP1* homolog in cyanobacteria also resulted in a complete loss of thylakoid formation (Westphal et al., 2001). The precise role of VIPP1 in the cycle of vesicle budding, migration, and fusion remains to be explored.

Xu et al. (2003) reported the finding of a mutant (*trigalactosyldiacylglycerol1* (*tgd1*)) defective in the biosynthesis of ER-derived thylakoid lipids. The ER pathway for the glycerolipid synthesis involves the return of DAG moieties from the ER to the chloroplast for synthesis of galactolipids and sulfolipids. When pulse labeled, the *tgd1* plant had reduced flux through the ER pathway of thylakoid lipid biosynthesis. The cloned gene encoded a permease-like protein which was localized to the chloroplast outer membrane, and was proposed to be a component of a lipid transfer complex.

Aminophospholipid ATPase1 (*ALA1*) in Arabidopsis is a member of at least 11 P-type ATPases and is homologous with yeast DRS2 and bovine ATPaseII aminophospholipid translocases (Tang et al., 1996; Ding et al., 2000; Gomes et al., 2000). *ALA1* complements the *drs2* phenotype of deficiency in PS internalization, and when overexpressed increased translocation of aminophospholipids in reconstituted yeast membrane vesicles (Gomes et al., 2000). One of the physiological functions of this putative aminophopholipid translocation seems to be linked to cold adaptation of plants since the Arabidopsis *ALA1* antisense plants showed chilling sensitivity.

Very long-chain fatty acyl derivatives of wax components have to be transported from their site of elongation and modification, the ER to the plasma membrane. After crossing the hydrophobic plasma membrane, they have to partition to the aqueous apoplast. Finally, the hydrophobic wax components must move through the hydrophilic cell wall matrix to reach the cuticle (Kunst and Samuels, 2003). Unpublished works by Samuels AL (Department of Botany, University of British Columbia, 6270 University Boulevard,

Vancouver, BC, Canada V6T 1Z4) suggested the involvement of a member of the ABC transporter family in the export of wax components from the cytosol.

The above review of lipid transport research in various organisms gives a general overview of the underlying issues and previous research on this topic. Although plant lipid metabolism in many ways differs from that of animal or unicellular organisms, the fundamental requirement for fatty acid movement is common for all eukaryotic and many prokaryotic organisms. The fact that the newly identified lipid transporters in plants have their counterpart genes in other organisms shows that at least some members of the transport system are of ancient origin and conserved throughout biology.

In this thesis three major aspects of fatty acid transport and metabolism are presented. First, Chapter 2 presents various kinetic labeling data to examine whether the export of FFA from plastids occurs via free physical diffusion or is mediated by a more complex system. Second, in Chapter 3 the question of movement of FA in the other direction, that is into the chloroplast is described and the compartmentation and the mechanism of exogenous FFA elongation activity in a plant cell is characterized. Third, Chapter 4 presents bioinformatics and reverse genetics approaches in search for plastid envelope lipid transporters. Finally, in Chapter 5, perspectives and directions for future research in this area are discussed.

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CHAPTER 2
ON THE EXPORT OF FATTY ACIDS FROM THE CHLOROPLAST

ABSTRACT

The model for export of fatty acids from plastids proposes that the acyl-ACP (acyl carrier protein) product of *de novo* fatty acid synthesis is hydrolyzed in the stroma by acyl-ACP thioesterases and the free fatty acid (FFA) released is then transferred to the outer envelope of the plastid where it is reactivated to acyl-CoA for utilization in cytosolic glycerolipid synthesis. Experiments were performed to assess whether the delivery of nascent FFA from the stroma for long chain acyl-CoA synthesis (LACS) occurs via simple diffusion or a more complex mechanism. The flux through the *in vivo* FFA pool was estimated using kinetic labeling experiments with spinach and pea leaves. The maximum half life for FFA in the export pool was $\leq$ 1 s. Isolated pea chloroplasts incubated in the light with [^{14}C]acetate gave a linear accumulation of FFA. When CoASH and ATP were present there was also a linear accumulation of acyl-CoA thioesters (plus derived polar lipids), with no measurable lag phase (< 30 s), indicating that the FFA pool supplying LACS rapidly reached steady state. The LACS reaction was also measured independently in the dark after *in situ* generated FFA had accumulated yielding estimates of LACS substrate-velocity relationships. Based on these experiments the LACS reaction with *in situ* generated FFA as substrate is only about 3% of the LACS activity required *in vivo* at the very low concentrations of the FFA export pool calculated from the *in vivo* experiment. Furthermore, bovine serum albumin rapidly removed *in situ* generated FFA from chloroplasts, but could not compete effectively for "nascent" FFA

substrates of LACS. Together the data suggest a locally channeled pool of exported FFA that is closely linked to LACS.

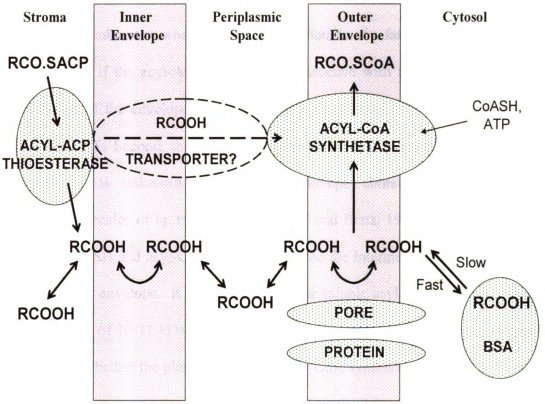
INTRODUCTION

De novo fatty acid biosynthesis in plants occurs mainly in the plastid (Ohlrogge et al., 1979). The immediate product, acyl-ACP thioester, may be used directly by plastid-localized acyltransferases for the synthesis of "prokaryotic" lipids that are assembled within the chloroplast. Alternatively the acyl moiety of the acyl-ACP can be exported to the cytoplasm where it is primarily incorporated into glycerolipids at the endoplasmic reticulum (eukaryotic pathway) (Roughan and Slack, 1982; Somerville and Browse, 1991). In leaves of plants such as Arabidopsis and spinach (16:3 plants) both pathways contribute about equally to the synthesis of leaf glycerolipids (Somerville and Browse, 1991), whereas in non-photosynthetic tissue of all plants and in leaves of 18:3 plants such as pea the eukaryotic pathway is the major pathway for glycerolipid synthesis (Ohlrogge and Browse, 1995).

The current model for export of fatty acids produced by *de novo* synthesis within the plastid proposes that acyl-ACP hydrolysis occurs in the plastid stroma by the action of acyl-ACP thioesterases, possibly at the inner leaflet of the inner envelope, and that the free fatty acid (FFA) released is transferred to the outer envelope of the plastid where it is reactivated to acyl-CoA for utilization in cytosolic glycerolipid synthesis (Figure 3). The chemical principal of this model was first proposed by Shine *et al.* (Shine et al., 1976). As subsequent experiments showed that ACP-dependent fatty acid synthesis was almost entirely a chloroplast function (Ohlrogge et al., 1979) this implied that the soluble acyl-ACP thioesterase was a stromal enzyme activity. Long-chain acyl-CoA synthesis

Figure 3. Schematic for fatty acid export from the site of *de novo* fatty acid synthesis in the chloroplast stroma to the cytosol.

Two simplified models are shown. The lower route is for simple diffusion of FFA and upper route is via a protein mediated export mechanism. RCO: long chain fatty acyl group, ACP: acyl carrier protein



(LACS) activity is associated with the chloroplast envelope (Roughan and Slack, 1977; Joyard and Stumpf, 1981) and more specifically with the outer envelope (Andrews and Keegstra, 1983; Block et al., 1983). In addition, the major product of fatty acid synthesis in isolated chloroplasts is FFA, unless ATP and CoASH are both present, when acyl-CoA becomes a major end product (Roughan et al., 1979; Roughan and Slack, 1981). *In vivo* confirmation that FFA is indeed an intermediate in fatty acid export from the plastid was accomplished by the use of ^{18}O labeling experiments (Pollard and Ohlrogge, 1999).

There are several unknowns when considering the details of a fatty acid export process. First, it is unclear if the acyl-ACP thioesterases associate with the inner leaflet of the inner membrane of the envelope, thus releasing the acyl group to the inner leaflet (as shown in Figure 3). Second, the vectorial nature of the long chain acyl-CoA synthesis (LACS) reaction is unknown. The outer envelope contains pores which can accommodate molecules of up to 8-10 kDa (Flugge and Benz, 1984). Thus metabolites such as ATP, CoASH and acyl-CoAs may diffuse into the intermembrane space between the inner and outer envelope. It is uncertain whether soluble acyl-CoA binding proteins, which have a MW of 10-11 kDa (Engeseth et al., 1996), will be able to move as freely. We do not know whether the plastid long chain acyl-CoA synthetase can bind FFA at the inner leaflet of the outer envelope, then transfer FFA to the outer leaflet for activation to acyl-CoA, or whether the transfer of FFA across the outer envelope is accomplished by other means, or if the active site of the long chain acyl-CoA synthetase is at the inner leaflet of the outer envelope. We note by way of analogy that pairs of proteins, one of

which is always an acyl-CoA synthetase, have been implicated in the vectorial import of fatty acids in *E. coli* (DiRusso et al., 1999), in yeast (Zou et al., 2003) and in animal cells (Schaffer and Lodish, 1994; Coe et al., 1999). Third, there appears to be no direct connections between the inner and the outer envelope of the plastid, although there may be some "contact points" (Douce and Joyard, 1990). Thus at the minimum we would expect that FFA will have to flip-flop across the inner envelope membrane, and then possibly disassociate into the periplasmic space to reach the long chain acyl-CoA synthetase at the outer envelope (Figure 3 lower route). Given these unknowns a major question is whether the movement of FFA out of chloroplasts is a transporter-facilitated process or involves free diffusion of the FFA intermediates. This is a debate which has parallels in other systems where FFA transport is required (Abumrad et al., 1998; Hamilton, 1998; Kleinfeld et al., 1997; Kamp et al., 2003). We report a variety of experiments designed to examine the question of FFA export from the plastid, using assays with intact leaves and chloroplasts.

RESULTS

The total in vivo pool of FFA in leaves is very small and implies a very short half life for the FFA pool involved in export from the chloroplast

Although it is generally known that labeled FFA are minor products when leaf tissue is incubated with acetate, the amount of FFA has not previously been quantified. We have shown that FFA are intermediates in fatty acyl group export from the chloroplast (Pollard and Ohlrogge, 1999) and to better understand this export process it was important to

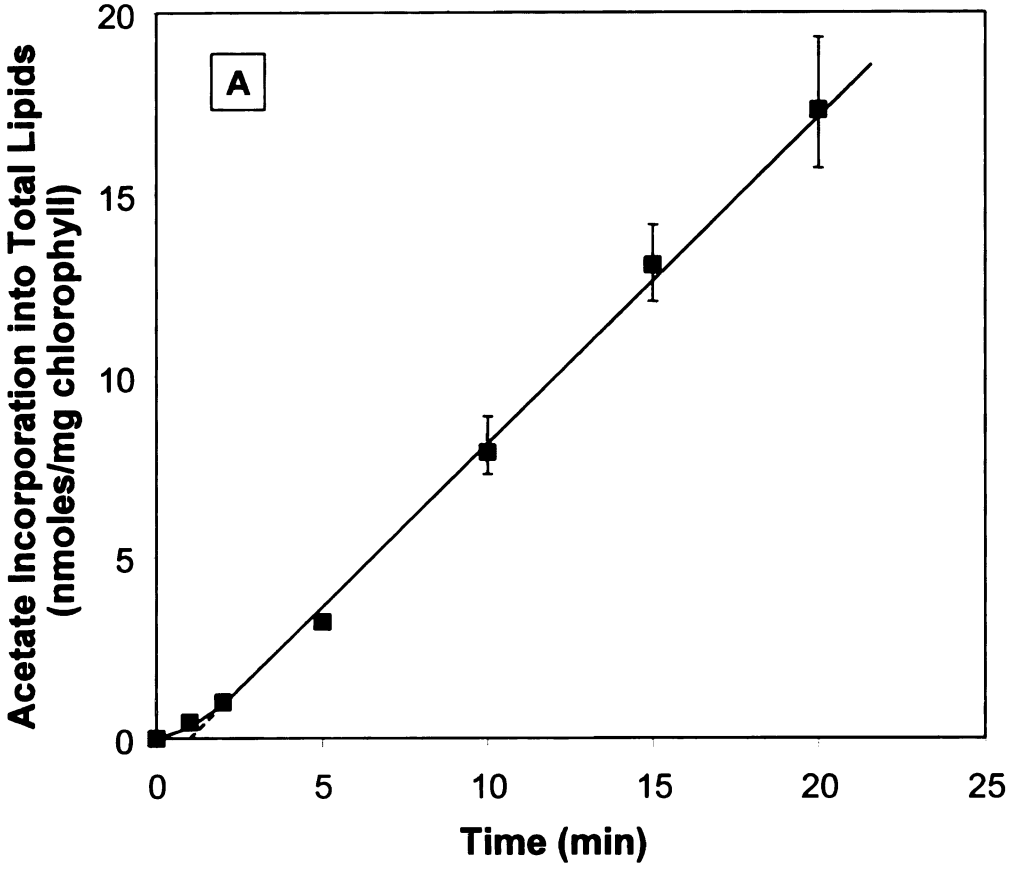
quantify this FFA pool. Figure 4 presents a time course for acetate incorporation into total fatty acids and into FFA by pea leaves over a 20 min period.

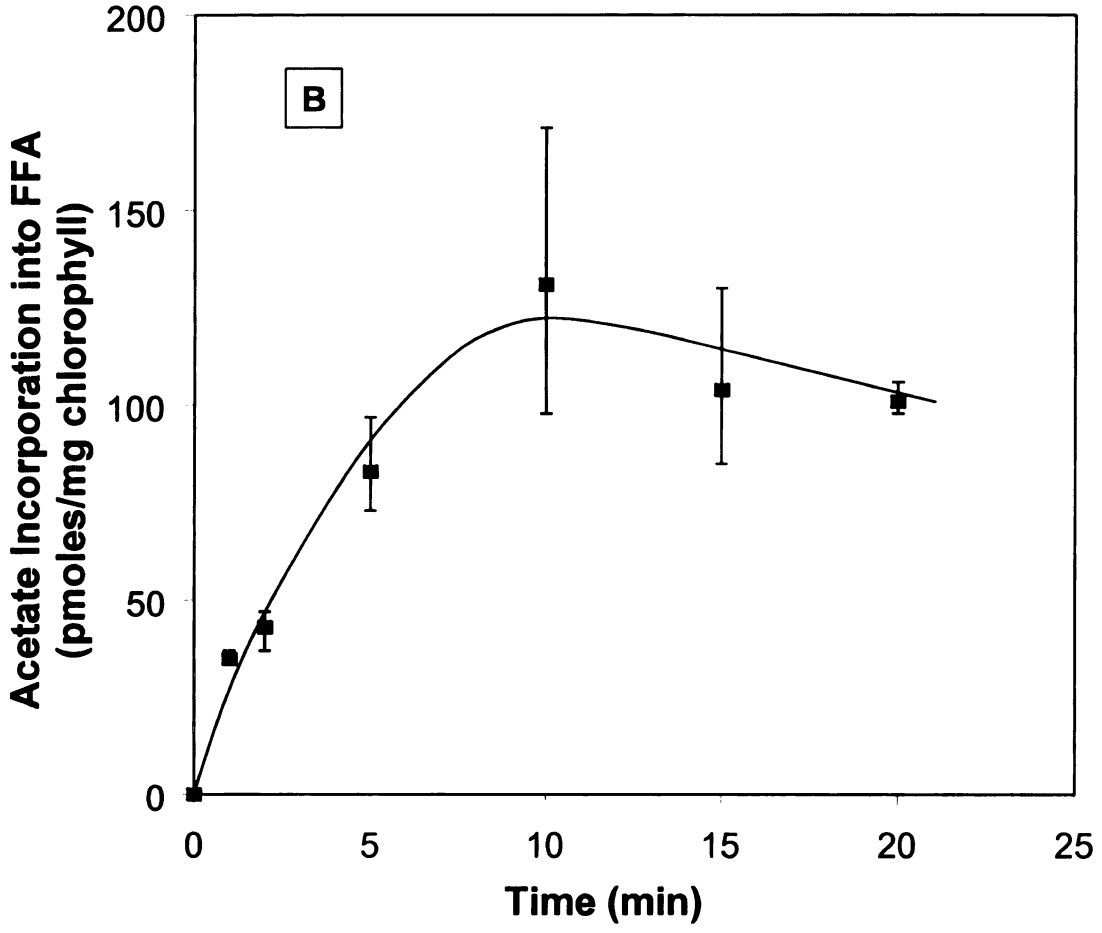
With expanding pea leaves a linear rate of lipid synthesis was established within 2 min (Figure 4A). At all time points the labeled total lipid extract from pea leaves contains 80-85% labeled fatty acids, mainly as phosphatidylcholine. Presumably the short lag phase indicates the time to reach the steady state balance between transport processes and pool filling in the biosynthetic utilization of acetate. To measure the FFA pool the leaf assays were quenched while still in the presence of substrate in the light (Figure 4B). Control extractions spiking unlabeled tissue with [^{14}C]oleic acid gave > 90% recovery of label.

In the experiment in Figure 4 the labeled FFA pool at 2 min was 4.3% of total labeled lipids and contained essentially only palmitic and oleic acids. In pea most of the fatty acids synthesized in the chloroplast are exported (ca. 90%). The turnover time of FFA during plastid export for pea can be estimated as follows. Let the steady state rate of lipid synthesis be 100 units per min, of which approximately 85 units are fatty acids. Therefore 76.5 units (90% of 85%) must pass through the FFA transfer pool for export to the eukaryotic pathway. At the two minute time point the steady state level of labeled FFA in the transfer pool has been reached, and because the steady state rate extrapolates back to the x-axis at 1 min (Figure 4A) the total amount of lipids synthesized is equivalent to about 100 units. Thus 100 units x 4.3% of labeled FFA are present (4.3 units). At two minutes we have a flux of 76.5 units per min through a transfer pool of 4.3 units. This gives a turnover time of 60 x 4.3/76.5 = 3.4 s and a $t_{1/2}$ value of 2.35 s ($t_{1/2}$ =

Figure 4. Time course for acetate incorporation into acyl lipids by pea leaves.

Pea leaf tissue was incubated with non-saturating concentrations of [^{14}C]acetate (0.105 mM). The data points are the average of triplicate determinations and error bars represent the range of values. Figure 4A shows acetate incorporation into total lipids by halved pea leaves over time. At each time point 80-85% of the label in total lipids was present as labeled fatty acid. Figure 4B shows acetate incorporation into the labeled FFA in the same experiment, and is measured as pmoles acetate incorporated/mg chlorophyll.


A
Acetate Incorporation into Total Lipids
(nmoles/mg chlorophyll)
Time (min)
0
5
10
15
20
25


B
Acetate Incorporation into FFA
(pmoles/mg chlorophyll)
200
150
100
50
0
0
5
10
15
20
25
Time (min)

turnover time x 0.693). A repeat of the time course with pea leaves gave the onset of steady state labeling within 1 min, while the labeled FFA pool at 2 min was 2.05 ± 0.26% (4 determinations), giving a $t_{1/2}$ value of 1.7 s. A similar time course and FFA pool analysis experiment was performed with spinach leaf strips (data not shown). A turnover time 0.96 s and a $t_{1/2}$ value of 0.66 s were determined.

These calculations for pea and spinach leaves represent estimated values for the FFA transfer pool turnover times that are an upper limit. The observed FFA pool may include the transient pool of FFA being exported from the plastid, FFA released from labeled acyl thioester pools during the heat quench step, plus FFA derived from other sources. The kinetics of labeled FFA appearance in the time course for pea leaves (Figure 4B) give us an indication that much of the labeled FFA pool is not a transfer pool. If the observed FFA pool were all the transfer pool then this pool would saturate by 2 minutes. Instead the pool continues to grow substantially. We speculate that some of the FFA pool actually derives from fatty acid synthesis at the damaged margins of the leaf strips in the assay. Given the approximations in the calculations it seems reasonable to state that the $t_{1/2}$ for FFA in any chloroplast export pool is about 1 s, or less, and possibly much less. Having established this limiting *in vivo* pool size we then examined the detailed relationship between products of fatty acid synthesis in isolated, intact pea chloroplasts. In pea, compared to spinach, much more of the fatty acid produced is exported from the chloroplast.

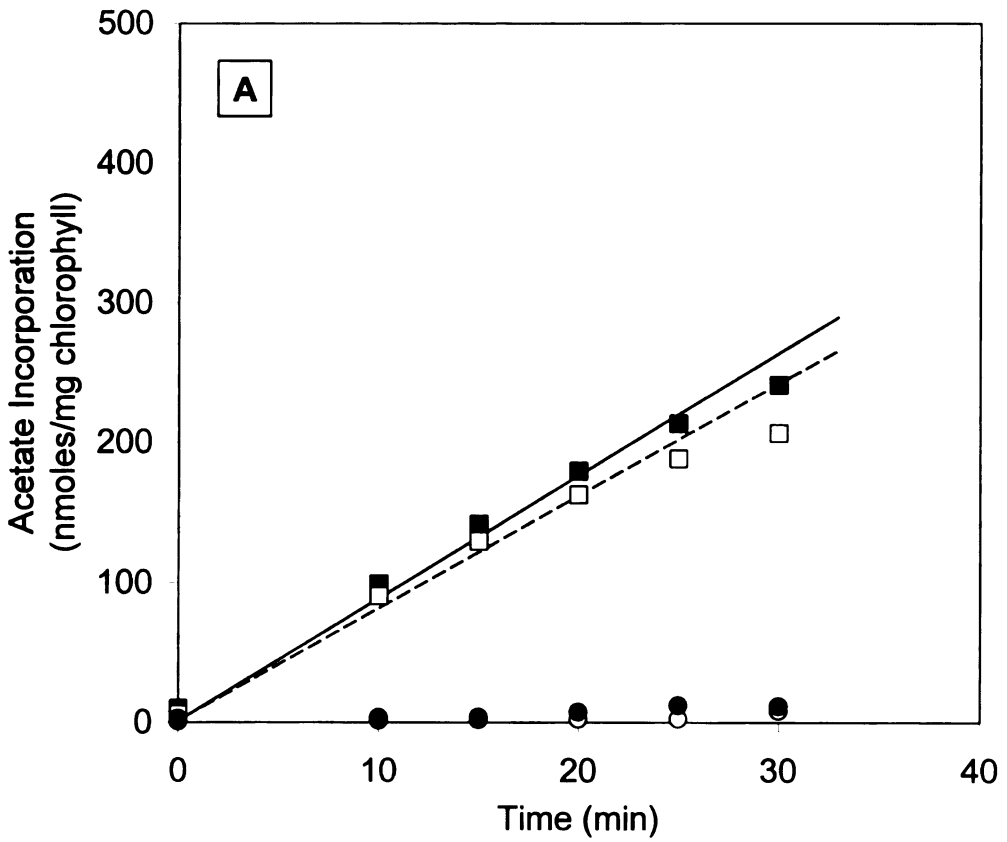
Time Course for Acetate Incorporation into Fatty Acyl Products by Isolated Pea Chloroplasts

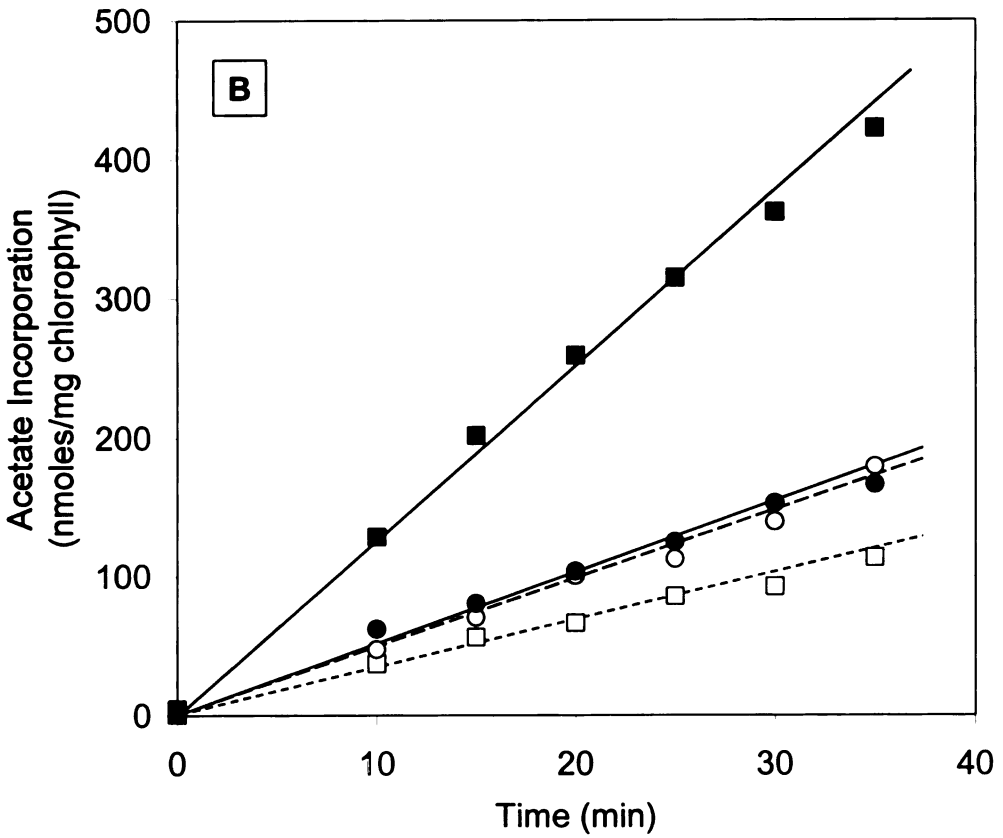
The synthesis of fatty acids from acetate by intact, illuminated chloroplasts can produce a variety of acyl products (Heinz and Roughan, 1983; Gardiner et al., 1984), depending on the plant species from which the chloroplasts are isolated and on the cofactors in the chloroplast incubation medium. In a minimal chloroplast incubation medium (no CoASH, glycerol-3-phosphate, nor UDP-galactose) the major product is free fatty acid (FFA). Smaller amounts of 1,2-diacylglycerol (DAG) (ca. 5-20%) and polar lipids (PL) (ca. 10-20%) have also been observed. When CoASH and ATP are added, up to 60% of the label may be found as acyl-CoA (Roughan and Slack, 1981).

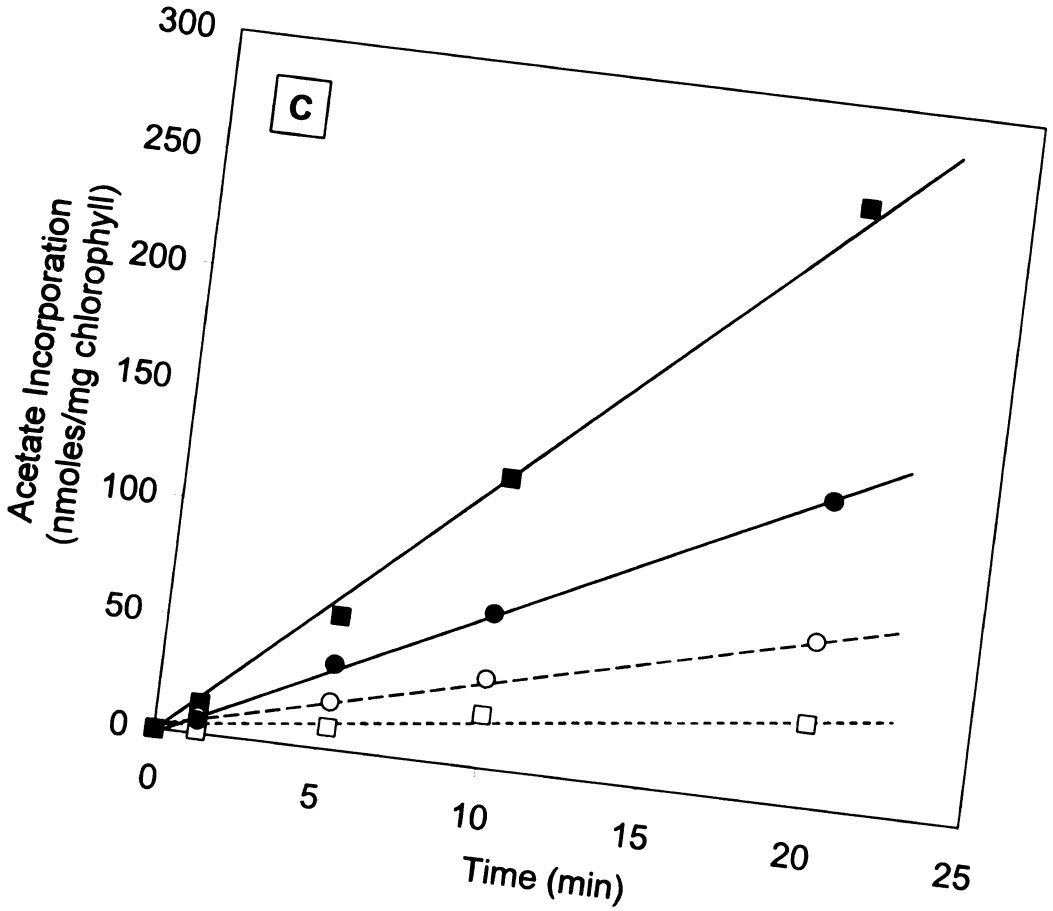
The incorporation of acetate into acyl lipids by pea chloroplasts is shown in Figure 5. In minimal chloroplast incubation medium the [^{14}C]acetate incorporation into total fatty acids was 15.5 pmoles fatty acid/s/mg chlorophyll (Figure 5A), but when CoASH and ATP were present this rate increased 1.4-fold to 21.5 pmoles fatty acid/s/mg chlorophyll (Figure 5B). In the minimal medium the major product was FFA (about 90% of total label). When CoASH and ATP were present up to 80% of the label was found as acyl-CoA plus polar lipids (PL). Figure 5B shows that over a 35 min period in the light with ATP and CoASH total fatty acid synthesis was linear, and that the appearance of the individual products, namely FFA (ca. 25%), acyl-CoA (ca. 40%) and total PL (ca. 40%) were also linear, with negligible lag phase detected. The labeled PL fraction was composed of about 60% phosphatidylcholine and 10% phosphatidylglycerol. To confirm the lack of a lag phase earlier time points were taken (Figure 5C). Any lag phase

Figure 5. Time course for acetate incorporation into fatty acyl products by pea chloroplasts.

In Figure 5A pea chloroplasts (40μg chlorophyll) were incubated under illumination in 0.2 ml basal medium with 2mM [^{14}C]acetate. For B and C 1mM ATP and 0.5mM CoASH were also added to the assay medium. The fractions assayed were total lipids (■), FFA (□), acyl-CoA (○), and total polar lipids (●).




B
Acetate Incorporation
(nmoles/mg chlorophyll)
500
400
300
200
100
0
0
10
20
30
40
Time (min)


C
300
250
200
150
100
50
0
0
5
10
15
20
25
Acetate Incorporation
(nmoles/mg chlorophyll)
Time (min)

required for the establishment of the steady state condition of product accumulation in the presence of CoASH plus ATP was less than 30 s. In Figure 5B or 5C if the bulk FFA pool were a precursor of acyl-CoA, which is subsequently used for PL synthesis, a measurable lag would occur in acyl-CoA plus PL accumulation. Thus there was no observable precursor-product kinetic relationship between labeled FFA and acyl-CoA. This implies that the FFA concentration to run the LACS reaction at steady state rate has been reached rapidly (< 30 s) and that the bulk labeled FFA pool accumulating in Figure 5B or 5C is not a precursor for the LACS reaction. The linear rates in Figure 5B or 5C further imply that neither the bulk FFA nor acyl-CoA are acting as feedback inhibitors for fatty acid synthesis by isolated chloroplasts. Our lag phase estimates are currently limited to > 10 s by the gentle mixing required for delicate organelles like chloroplasts, for pool filling from acetate through to end products of fatty acid synthesis, and quench times.

The Long Chain Acyl-CoA Synthesis (LACS) Reaction in the Dark Utilizing in situ Generated FFA

When FFA is exported from the plastid it is converted to acyl-CoA for utilization in cytosolic reactions of lipid biosynthesis. The LACS reaction has previously been studied after preparation of chloroplast envelope vesicles and addition of FFA substrate (Joyard and Stumpf, 1981). In this study we investigated the LACS reaction in intact pea chloroplasts with *in situ* generated FFA. Figure 6 presents the effect of switching off the light after 15 min of incubation of chloroplasts in media containing no CoASH during the light period but added at the onset of the dark period, or in media containing CoASH during both light and dark periods. As observed by many researchers (Browse et al.,

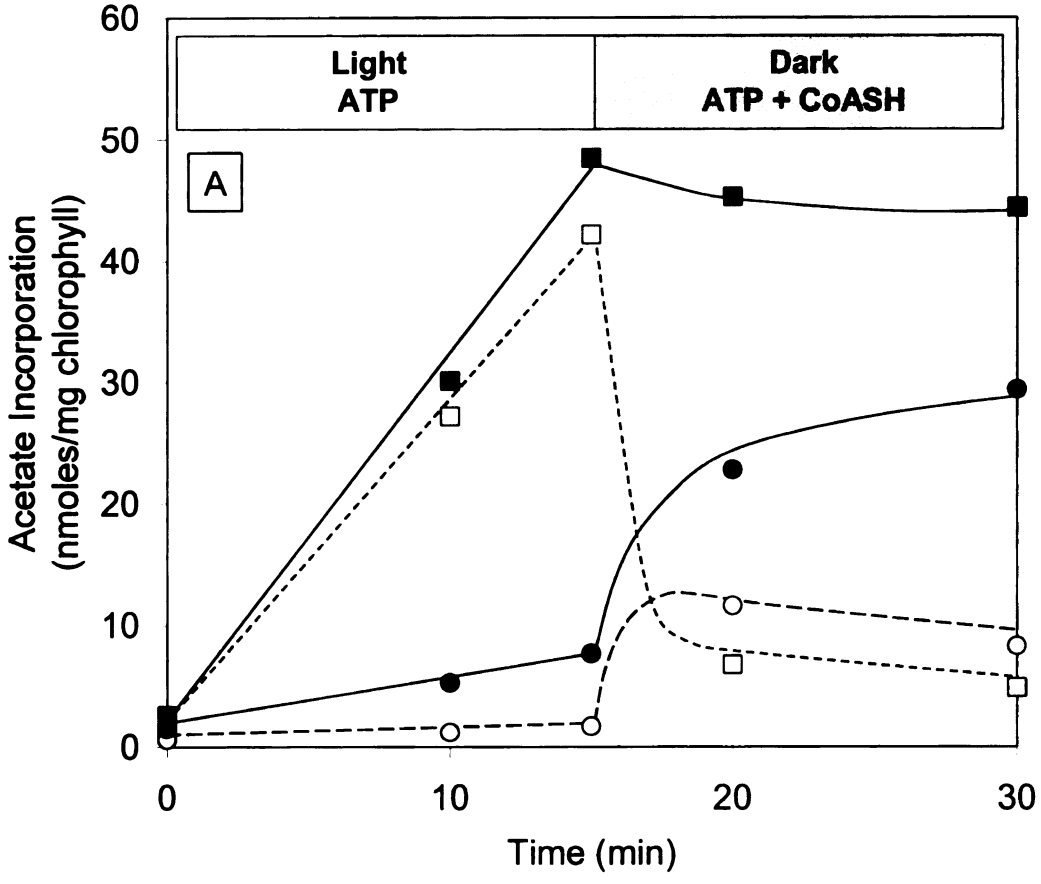
1981; Roughan et al., 1980) total fatty acid synthesis was completely halted in the dark. What is noteworthy is the very different behavior of the individual acyl pools during the dark period. In the absence of CoASH the major fatty acid product in the light was FFA (Figure 6A). When CoASH (0.5mM) was added at the transition to darkness there was a very rapid conversion of FFA to acyl-CoA and its metabolites (PL). The initial rate of FFA depletion (from 15 to 20 min) was at least twice the rate of total fatty acid synthesis in the light. By contrast, in Figure 6B the initial rate of FFA depletion on transition to darkness was less than 30% that of the rate of fatty acid synthesis in the light. The combined acyl-CoA and PL accumulation had essentially stopped in the dark period.

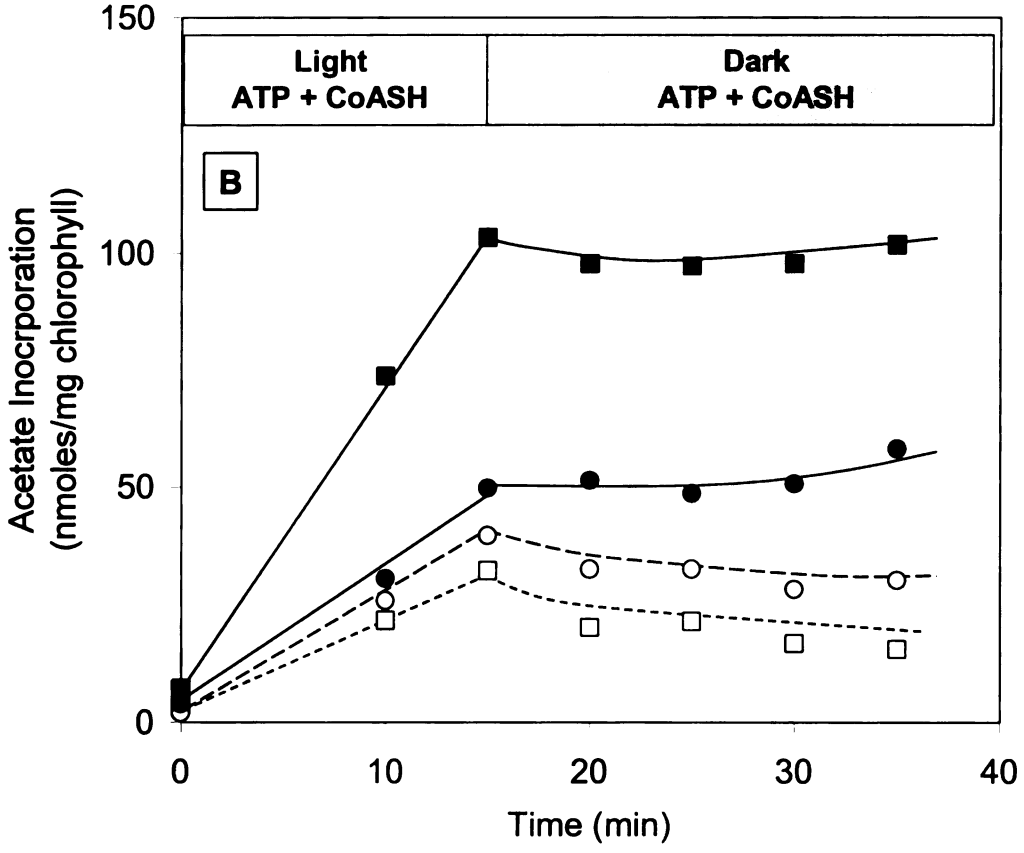
Because the LACS reaction with *in situ* generated FFA was very fast in Figure 6A this experiment was repeated with additional measurements immediately after the light to dark transition (Figure 6C). There were variations between five independent experiments but upon the dark transition with coincident addition of CoASH, about 25-50%, and sometimes >50% of the FFA label was removed by the LACS reaction in the first 30 s. Thus the LACS reaction in chloroplasts has the capacity to run at a rate over 10 times that of fatty acid synthesis. However, the LACS reaction did not rapidly go to completion. Inspection of Figure 6C shows that disappearance of FFA does not follow simple first order kinetics. There are at least two components to the disappearance of FFA; a rapid rate of depletion accounting for at least 60-70% of the labeled FFA but sometimes up to 80-85% depending on experiment, and a slow decay for the remainder.

Evidence for Distinct Kinetic Pools of FFA in Chloroplast Assays

Figure 6. Depletion of FFA pool after transition from light to dark.

Pea chloroplasts (40 μg chlorophyll) were incubated with 2mM [^{14}C]acetate under illumination in 0.2 ml basal medium containing 1 mM ATP for 15 minutes. After 15 minutes the lights were removed and the assay continued. Panel A and C: CoASH (0.5mM) was added at the moment of transition into dark. Panel B: CoASH (0.5mM) was added from the beginning of incubation in the light. The data points are average of 3 independent determinations. In panel A and B the fractions assayed were total lipids (■), FFA (□), acyl-CoA (○), and total polar lipids (●). Panel C shows only the reaction after the CoASH addition on transfer to the dark, and the fast and slow rates of FFA depletion are indicated. In panel C the fractions assayed were FFA (□), acyl-CoA (○), and acyl-CoA plus polar lipids (▲).


Light
ATP
Dark
ATP + CoASH
A
Acetate Incorporation
(nmoles/mg chlorophyll)
60
50
40
30
20
10
0
0
10
20
30
Time (min)


Light
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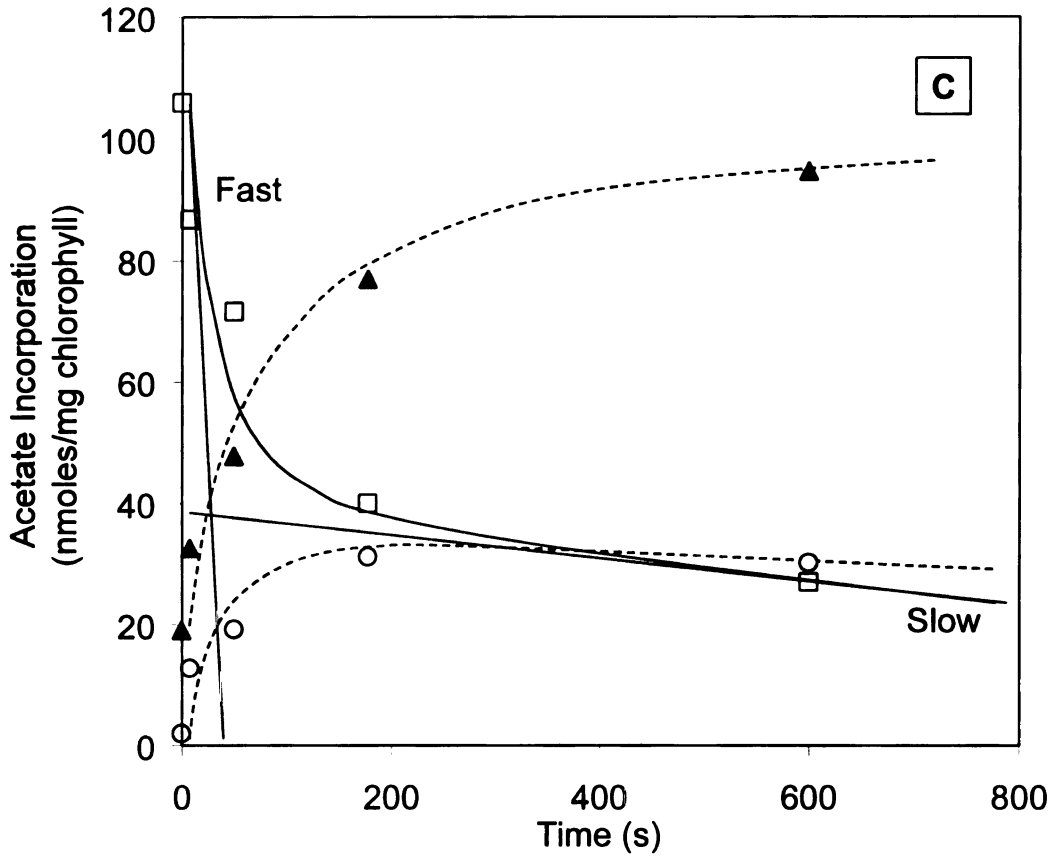
C
Fast
Slow
Acetate Incorporation
(nmoles/mg chlorophyll)
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0
20
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120
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200
400
600
800

Based on Figure 6A we know the LACS reaction is highly active in the dark and therefore the slow rate of LACS in Figure 6B appears somewhat paradoxical, especially since in Figure 6A the amount of labeled FFA at the dark transition is 4.7 nmoles/mg chlorophyll while in Figure 6B it is still 3.5 nmoles/mg chlorophyll. This paradox is also seen in Figure 5B with the linear accumulation of FFA up to 12 nmoles/mg chlorophyll in the presence of ATP and CoASH, and is best explained by multiple FFA pools. The results shown in Figures 5 and 6 reveal three kinetically distinct pools of FFA products. In all of these pools FFA is assumed to be associated with the chloroplasts and partitioned largely or completely into membranes. These pools are (A) the very small FFA pool supplying the LACS reaction in the light in the presence of CoASH and ATP (Figure 5B and 5C), (B) a bulk FFA pool that builds up if the LACS reaction cannot run in the light because of the absence of CoASH but which is accessible to LACS when CoASH and ATP are present (Figure 5A), and (C) a bulk FFA pool (about 20 -30% of total label) that is not readily accessible to LACS reaction either in the light or the dark in the presence of ATP and CoASH. If the appearance of acyl-CoA in assays run in the presence of ATP and CoASH in the light is indeed catalyzed by the same enzyme that catalyzes the LACS reaction in the dark, then bulk pool (B) will provide substrate for pool (A). Thus in the light in the presence of ATP and CoASH, newly synthesized FFA rapidly moves through pool (A), or accumulates in a linear fashion in pool (C) (Figure 5B and 5C). Stopping fatty acid synthesis by placing the assay in the dark does not cause much additional synthesis of acyl-CoA and PL because pool (B) is absent (Figure 6B). In the absence of CoASH during fatty acid synthesis in the light, both pool (B) and pool (C) accumulate in a linear fashion, and when the assay is placed in the dark with CoASH,

pool (B) is available for a rapid LACS reaction (Figure 6A and 6C). The physical nature of these distinct FFA pools becomes an important question. Presumably *in vivo* only pool (A) is present. Pool (B) is artefactual in that *in vivo* the LACS reaction is not cofactor limited, but as we will discuss later serves as a useful model if FFA is allowed to diffuse freely within the organelle. The LACS reaction of pool (B) FFA would be a mass action driven activation of FFA. Pool (C) is an artefact, perhaps as a consequence of a population of damaged chloroplasts.

The Dependence of the LACS Reaction in the Dark on the Concentration of in situ Generated FFA

The rapid depletion of FFA by the LACS reaction seen in Figure 6C will require a series of FFA transport steps (membrane flip-flops and association and disassociation steps) to deliver the FFA to the site of the LACS reaction. We do not know the distribution of FFA within the chloroplast, or the orientation of the LACS active site in the outer envelope, so we cannot speculate on the complexity of these steps. However, if we approximate the FFA fast depletion reaction to a single (averaged) time constant for exponential decay of pool (B) then that time constant is approximately 30 s, or sometimes less. The concentration dependence of the FFA depletion is shown in Figure 7, using a 30 second dark LACS reaction assay. At the levels of FFA we allowed to accumulate in the light preincubation period, the rate of the dark LACS reaction was essentially proportional to FFA concentration and thus not saturated with respect to FFA substrate.

Accessibility of Chloroplast FFA Products to Sequestration by BSA in the Dark

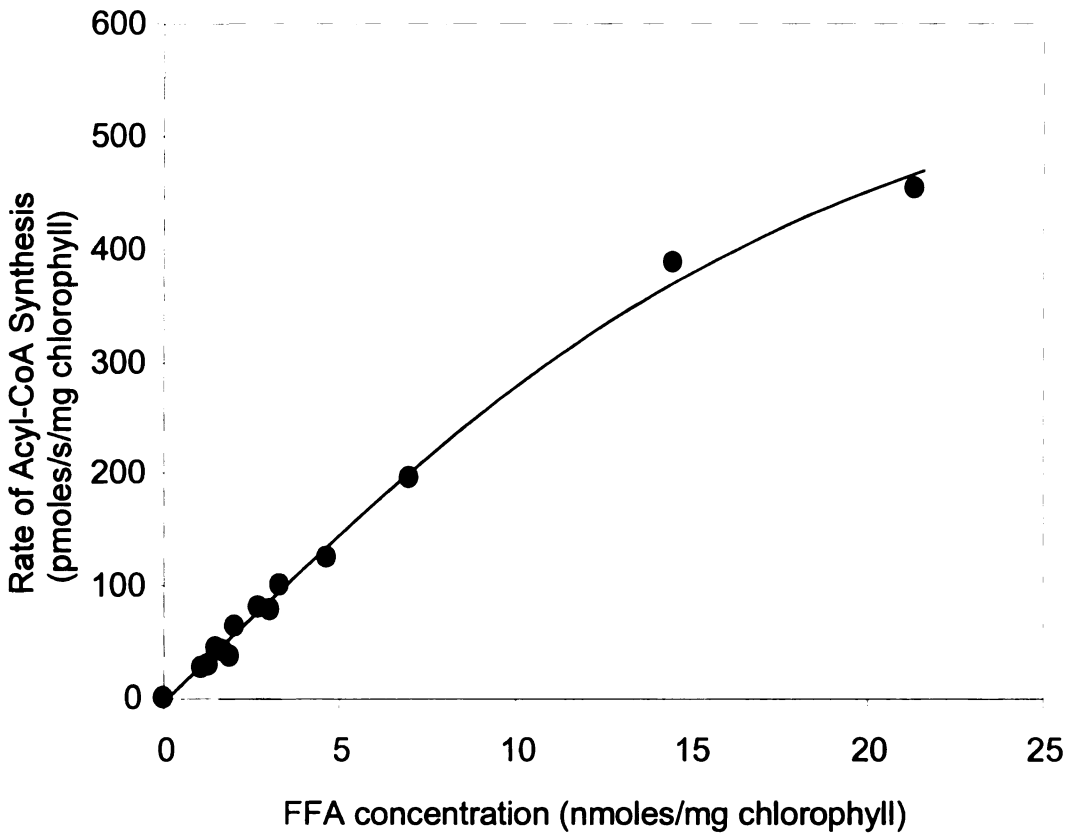
BSA is well known for its ability to bind FFA (Parks et al., 1983; Hamilton et al., 1984). We have used this FFA-binding capacity in competition experiments with chloroplasts. Chloroplasts that were pre-loaded with *in situ* generated [^{14}C]FFA were incubated with different concentrations of defatted BSA for 5 min in the dark and centrifuged to separate the chloroplast pellet from the media. Radiolabeled lipids in the media and in the chloroplast pellet were assayed. At the beginning of the assay 80-85% of the total labeled product was FFA and >85% of the total labeled lipid was associated with the chloroplast. However when the chloroplasts containing labeled FFA were mixed with increasing concentrations of BSA, increasing amounts of FFA were found in the supernatant. This redistribution saturated at about 80% of the label released into the medium at a BSA concentration of 0.3 mg/ml (4.5 μM).

The concentration of labeled FFA produced *in situ* in the assay was 2.5 μM. Next the rate of FFA transfer from the chloroplasts to the BSA was examined by varying the co-incubation time at 0.5mg/ml BSA (Figure 8). Over 90% of the labeled FFA partitioned into the media bound to the BSA within 45 s. This was the quickest practical time to conduct transfers and centrifugation. Thus the $t_{1/2}$ for FFA sequestration by BSA is about 10 s or less. Presumably the FFA:BSA binding reaction is mediated via BSA binding to the outer leaflet of the outer envelope and requires FFA to be present in the outer leaflet and to move through to the outer leaflet rapidly from other sites in the chloroplast.

BSA Competes with LACS for the Bulk in situ FFA Pool but Is Not As Effective for the Nascent FFA Pool

Figure 7. FFA concentration dependence of the LACS reaction.

Isolated pea chloroplasts were incubated with [^{14}C]acetate under illumination in 0.2 ml basal medium containing 1 mM ATP for varying times in the absence of CoASH and then lights were removed. One set of assays was quenched at this point to determine the FFA pool accumulated. To a second set of assays CoASH (0.5mM) was added at the moment of transition into dark. After another 30 seconds in the dark the reaction was stopped by freezing immediately with liquid nitrogen and the FFA assayed to assess its depletion by the LACS reaction.



Because BSA can rapidly and almost completely remove FFA generated *in situ* by chloroplasts (Figure 8), experiments were conducted to measure the rate of the LACS reaction with FFA substrate bound to BSA. FFA was first generated *in situ* in the presence or absence of BSA for 15 min, then CoASH was added in the dark (Figure 9). Comparing the initial rates for the LACS reaction the control reaction utilizing FFA bound to the chloroplast was at about 20 times faster than the rate for FFA substrate sequestered in the medium bound to BSA. This difference may be greater still if the results are normalized to the same assay concentration of FFA generated *in situ*. The data from Figure 9 indicate that the release of FFA to the chloroplast by BSA is a relatively slow step.

The results of a continuous labeling assay under light in the presence and absence of BSA are shown in Figure 10. When ATP, CoASH and BSA are present from the beginning of the assay, LACS and BSA are expected to compete for *de novo* synthesized "nascent" FFA from the export pool (A). If the FFA diffuses out to the outer envelope, BSA may have a chance to bind a fraction of the FFA and thereby increase the proportion of FFA in the total product. As observed in a previous study (Johnson et al., 2002) BSA stimulated total fatty acid synthesis activity 1.6 fold (average of 4 different experiments, data not shown). With the addition of BSA the acyl-CoA product increased from 26% to 37% and that of PL decreased from 52% to 41% while FFA level remained unchanged at 22%. The fact that BSA was not able to increase the proportion of FFA in the products again argues against the idea that there was a bulk freely-diffusing pool of FFA during the

Figure 8. Sequestration of FFA by binding to BSA.

Isolated pea chloroplasts were first loaded with *in situ* generated [^{14}C] FFA by incubation in minimal media with [^{14}C]acetate for 15 min in light. Upon transition to the dark BSA (0.5 mg/ml) was added to the assay medium which was then centrifuged (16,000x g) for 15 s at various time points. Supernatant was aspirated and collected and the transfer reaction was terminated by quenching both the supernatant and pellet immediately with liquid nitrogen. The fractions assayed were FFA (■), acyl-CoA (○), and polar lipids (□).

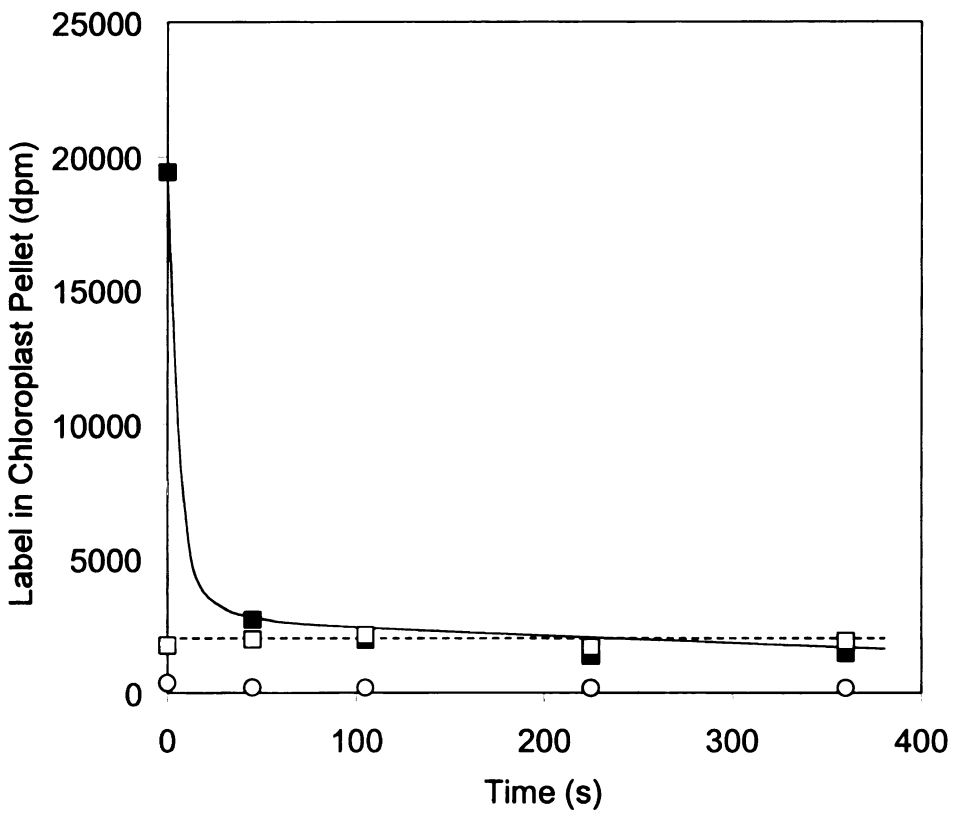
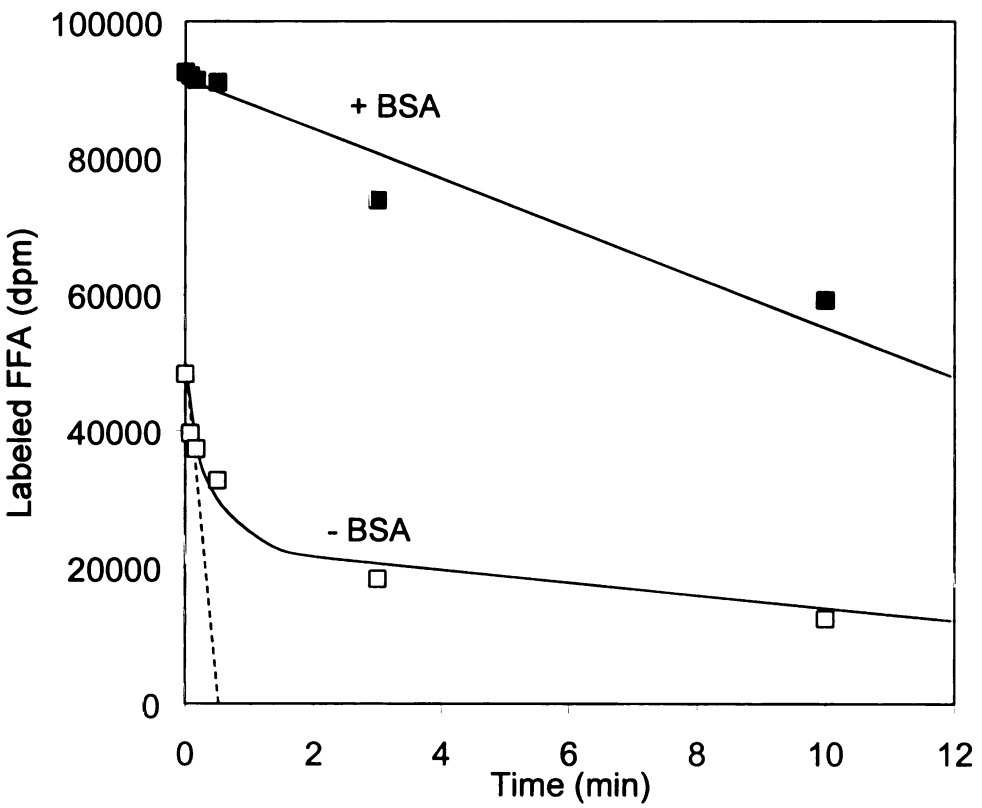


Figure 9. Competition between LACS and BSA for *in situ* generated FFA.

Isolated pea chloroplasts were first loaded with *in situ* generated [^{14}C] labeled fatty acids by incubation in minimal media containing [^{14}C]acetate as substrate without CoASH but in the presence (■) or absence (□) of 0.5 mg/ml BSA for 15 min in light. Upon transition to dark, CoASH (0.5mM) was added. Reactions were terminated after further incubations with CoASH for various times. Label in FFA was measured. The initial rate in the presence of BSA was 4050 dpm/min, and in the absence of BSA (dotted line) 77000 dpm/min.



process of FFA export from the chloroplast and suggests that LACS has "prior" access to exported FFA before it diffuses out to the outer leaflet of the outer envelope membrane.

DISCUSSION

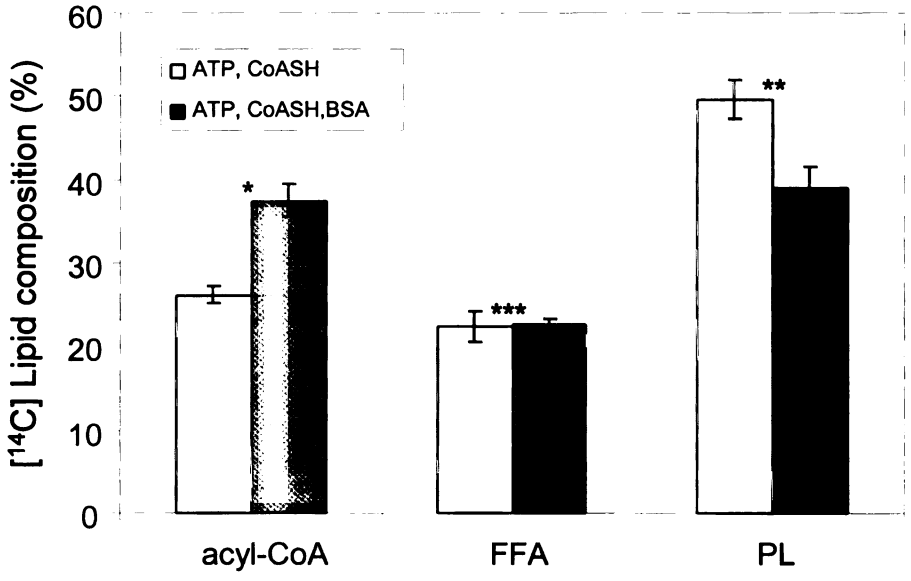
The Endogenous Free Fatty Acid Pool

The principal products of *de novo* fatty acid synthesis exported from most chloroplasts are palmitate and oleate (Ohlrogge and Browse, 1995). In our previous study of leaf fatty acid synthesis from ^{18}O-acetate (Pollard and Ohlrogge, 1999), the fatty acyl export from the plastid was demonstrated to involve a hydrolytic step to produce a free carboxylate anion prior to the fatty acid reactivation for incorporation into eukaryotic lipids. This was an expected result but had not been previously demonstrated *in vivo*. The confirmation makes consideration of FFA transport mechanisms in plastids a relevant physiological problem. An immediate question to address is the size of this FFA transport pool. In all carefully conducted labeling studies of plant tissues only a very small radiolabeled FFA fraction is observed in experiments with [^{14}C]acetate. From our kinetic analysis in this study we estimate that the $t_{1/2}$ for FFA in any plastid export pool is ≤ 1 s.

At this point this upper limit placed on the $t_{1/2}$ value for FFA is not sufficient to rule out any mechanistic possibility. At one extreme it is instructive to consider the stoichiometry

Figure 10. Competition between LACS and BSA for "nascent FFA" in fatty acid synthesis assays.

Isolated pea chloroplasts were incubated with [^{14}C]acetate under illumination in a basal incubation medium containing 1mM ATP and 0.5 mM CoASH, in the presence or absence of 0.5 mg/ml BSA. Samples were collected at 5, 10 and 20 minute time points and the percentage of total labeled lipids present in FFA, acyl-CoA and polar lipid fractions was analyzed. Values represent the means of 15 measurements from 4 independent experiments, with standard deviations shown. Student t-test comparing control and BSA added samples showed that differences are significant for acyl-CoA* ($p < 0.001$) and PL** ($p<0.01$) but are insignificant for FFA*** ($p > 0.5$).



for any channeled, protein-mediated transport process. Heber and Heldt (1981) have estimated that the inner envelope protein concentration is 200-300 μg/mg chlorophyll. The endogenous rate of fatty acid export is about 15-55 pmoles/s/mg chlorophyll (see below). Assuming a transport protein of 60 kDa, with a 1:1 protein:FFA binding stoichiometry and a 50% occupancy of binding sites, then if the average transfer time is about one second, the transfer protein concentration is 2-6 μg/mg chlorophyll, or about 0.5-3% of the inner envelope protein. Thus the time constant is consistent with the possibility of a channeled transport system. Should there be a FFA transporter protein (passive or active) in the inner envelope, it is probable given the 2-10 nm periplasmic space between the two envelopes that it may be in direct contact with the acyl-ACP thioesterase at the stromal interface of the inner envelope and with the LACS protein on the outer envelope (Figure 3 upper route).

At the other extreme, consider the process of free diffusion from the plastid. The possible steps are shown in Figure 3 lower route. A debate between a facilitated process and free diffusion for FFA uptake by various types of animal cells has persisted for many years (Abumrad et al., 1998; Hamilton, 1998; Kamp et al., 2003; Kleinfeld et al., 1998), and some points are instructive to the issues of FFA export from the plastid. Very fast rates of FFA flip-flop ($t_{1/2} \leq 25$ ms) are observed for small (0.025 μm diameter) and large (0.1 μm diameter) unilamellar PC vesicles, (Hamilton, 1998; Kamp et al., 1993; Kamp et al., 1995). However, Kleinfeld *et al.* (1997) pointed out that there is an inverse relationship between $t_{1/2}$ for FFA flip-flop and the radius of curvature of the liposomes

studied. In giant unilamellar vesicles (>0.2 μm diameter) $t_{1/2}$ values of 0.7-7 s were measured for FFA flip-flop, values that were later found to be similar for erythrocyte ghosts (Kleinfeld et al., 1998). Chloroplasts typically have diameters of 1-4 μm. An important point is that there is no direct physicochemical measurements for FFA flip-flop or binding and desorption from liposomes composed primarily of galactolipids, such as to mirror the composition of the chloroplast inner envelope or the inner leaflet of the outer envelope. In the absence of such measurements it is difficult to speculate whether the physical rates of FFA flip-flop and membrane desorption might be adequate to account for the rate of FFA export from the plastid, or for any movement of FFA into and from thylakoids. Even with a series of physical measurements the chloroplast is a complex organelle to model when compared to liposomes and erythrocyte ghosts, in that the envelope is a double membrane. In addition within each chloroplast there is the massive endomembrane system of thylakoids that accounts for >90% of the organelle's membrane and which might sequester FFA. Furthermore, in the light the pH of the stroma is about 8.5 whereas the pH of the cytosol is about 7.5. Thus freely diffusing FFA will preferably partition to stromal facing leaflets in chloroplast membranes, that is, to those of the inner envelope and thylakoids.

FFA Pools in Incubations with Intact Chloroplasts

In assays with spinach chloroplasts Roughan et al. (1980) noted that when chloroplasts were incubated in the light for 20 min in basal medium and then transferred to the dark for 20 min with CoASH and ATP, the FFA labeled product was reduced by half with the

concomitant appearance of acyl-CoA as the major product (or PC when microsomes were also added). These assays were not run as kinetic assays, since their purpose was product identification. The implication in this and other papers and reviews is that the acyl-CoA formed upon addition of ATP plus CoASH is derived from the "bulk" FFA pool (Johnson et al., 2002). Because our kinetic analysis of in vivo leaf assays indicated that the $t_{1/2}$ for FFA in any plastid export pool is $\leq$ 1 s this very small pool is inconsistent with the large FFA pools seen in assays with isolated chloroplasts, and particularly with their being functional transport intermediates. We have investigated this apparent contradiction and showed there is not in fact an inconsistency if the total FFA produced in chloroplast assays is described as the sum of several distinct FFA pools.

The results from our kinetic assays with intact chloroplasts (Figure 5 – 10) are interpreted in terms of three fates (or pools) for FFA. A very small FFA pool (A) that provides "nascent" FFA for LACS is implicated by the lack of precursor-product kinetics between total FFA and acyl-CoA, as shown in Figure 5B. This pool size (i.e. $t_{1/2}$ value) is too small to measure accurately but we postulate it is analogous to the *in vivo* situation. The LACS reaction is not feedback inhibited by continued accumulation of acyl-CoA. The bulk accumulating FFA (Figure 5A) can be best described as a combination of two pools, neither of which represents a physiological situation. One pool (B) is a LACS-accessible pool which fills when the LACS reaction cannot function because of lack of cofactors. The other pool (C) is present in all assays, represents 20 to 40% of the total labeled fatty

acids and is not rapidly accessible to LACS. Because Pool C is likely a function of damaged chloroplasts, it is not discussed further.

At saturating concentrations BSA rapidly removes FFA from the chloroplast ($t_{1/2}$ < 10 s) (Figure 8). This allowed us to test it as a competitor against the putatively channeled LACS reaction, since the time constant for FFA removal from the chloroplast by BSA was equal to or less than that for the independent LACS reaction. In addition, once FFA is bound to BSA, the LACS reaction is reduced at least 20 fold (Figure 9). When fatty acid synthesis occurs simultaneously in the presence of ATP, CoASH and BSA, BSA does not inhibit the appearance of acyl-CoA products by sequestration of the exported FFA (Figure 10). A simple explanation can be advanced for this result. Since we believe the BSA binding of FFA is a physical transfer process it is likely that the rate of removal of FFA from chloroplasts by BSA is proportional to the bulk FFA concentration, which initially is about 2.5 μM (Figure 8). In intact chloroplasts BSA cannot penetrate the outer envelope, and therefore must load FFA from the outer leaflet of the outer envelope or from the very low amounts of FFA partitioned into the medium. We can estimate pool (A), the pool of FFA required to sustain the channeled LACS reaction, as filling within 30 s or 1 s (using the *in vitro* or *in vivo* estimates), which gives the bulk concentration of FFA product in the chloroplast assay as <35 nM or <1 nM. With either calculation, on simple kinetic grounds BSA should not effectively "intercept" FFA in pool (A). That is, the lack of effect by BSA on products of continuous chloroplast assays containing ATP and CoASH (Figure 10) is consistent with our pool (A) interpretation.

LACS Activity in Isolated Chloroplasts and a Comparison with Leaf Tissue Labeling Experiments Suggest a Channeled FFA Export Pool

The independent LACS reaction was measured in isolated intact chloroplasts by first loading chloroplasts *in situ* with FFA accumulated from *de novo* fatty acid synthesis in the light after which the LACS reaction was run in the dark by adding CoASH (Figure 6A, 6C). The data in Figure 7 show that the initial LACS rate for a 15 min loading period is not saturated with respect to FFA (it is saturated with respect to ATP and CoASH) and therefore the depletion of pool (B) can be very approximately described by a first order rate equation $k_{(lacs)} \cdot [FFA]_B$. Because *in situ*-generated FFA can be rapidly removed by BSA (Figure 8) pool (B) must be freely diffusible. We also know it is mostly associated with the chloroplasts (Figure 8) unless the reaction mixture contains FFA scavengers. However, we cannot be certain that FFA in pool (B) has partitioned into the thylakoid membranes. Thus the intra-organelle location of FFA pool (B) may be complex and FFA depletion in the dark by LACS may reflect complex kinetic parameters, that is, various FFA diffusion rates (lateral diffusion, flip-flop, partitioning between membrane and media), LACS enzymatic properties, possible LACS feedback inhibition and the possibility of multiple LACS enzymes with different K_m values etc. As we assert below that pool (A) represents a channeled system, pool (B) may be considered analogous to what might happen *in vivo* if there was no channeling (eg. in a mutant).

The endogenous rate of fatty acid synthesis in leaves is of the order of 1-2 μmole C2 units/h/mg chlorophyll (Pollard and Ohlrogge, 1999; Browse et al., 1981; Bao et al., 2000). This translates to about 15-55 pmoles C18 fatty acids/s/mg chlorophyll for plastid export, assuming that 50-90% of newly synthesized fatty acids are exported. In our pea chloroplast assays with exogenous acetate (Figure 5B) acyl-CoA synthesis (acyl-CoA plus PL) reached a steady state rate of 17.5 pmoles C18 fatty acids/s/mg chlorophyll in less than 30 s. This value is lower than the LACS V_{max} measured by Joyard and Stumpf (1981). These authors characterized the LACS reaction in envelope membrane vesicles isolated from spinach leaf chloroplasts. Assuming that the protein to chlorophyll ratio is 20:1 and that envelope protein accounts for about 1% of total plastid protein their V_{max} for 18:1-CoA formation was 85-140 pmoles/s/mg chlorophyll. In Figure 6C the total rate of fatty acid synthesis is 16.7 pmoles fatty acid/s/mg chlorophyll during the light period, and when the dark reaction is run the initial LACS rate is 220 pmoles fatty acid/s/mg chlorophyll. This is 13-fold greater than the rate of fatty acid synthesis. The maximum rate of the dark LACS reaction is estimated from Figure 7 as 670 pmoles fatty acid/s/mg chlorophyll. We estimate that our assay concentration of FFA to drive the LACS reaction of intact pea leaf chloroplasts at half maximum velocity as 3 μM, which is well below the 200 μM K_m reported by Joyard and Stumpf (1981) for oleic acid activation by isolated chloroplast envelopes from spinach. However comparing bulk FFA concentrations between assay systems for envelope vesicles and chloroplasts causes inconsistencies, particularly with respect to the ratio of FFA to membrane lipids etc. The numerical comparison should not be taken literally, but the comparison, albeit for

different plant species, does suggest that the LACS enzyme has a much greater affinity for FFA than measured by studies with isolated envelope vesicles.

A more significant comparison is to assess the LACS rates predicted by Figure 7 when using estimates of the pool size of FFA available for the reaction. For the chloroplast continuous labeling assays in the light the steady state rate (Figure 5B, C) is established within 30 s. At this time point the assay concentration of FFA is 0.17 nmoles/mg chlorophyll (Figure 5B, C), which, according to Figure 7 should sustain a LACS rate of 4.6 pmoles/s/mg chlorophyll. However, the steady state rate of acyl-CoA production of 17.5 pmoles/s/mg chlorophyll has been established. Clearly, LACS operating on a freely diffusing FFA pool within the chloroplast can not explain the onset of the steady state condition. Furthermore, if we take the $t_{1/2}$ value of one second, derived from the *in vivo* experiments, as the time to fill the transfer FFA pool then the *in vivo* FFA transfer pools are about 15-55 pmoles C18 fatty acids/mg chlorophyll. These FFA concentrations would support 0.4-1.4 pmoles C18 fatty acids/s/mg chlorophyll according to the concentration dependence curve for chloroplast LACS activity shown in Figure 7. The discrepancy between acyl-CoA synthesis rates in chloroplasts when fatty acid synthesis is run in the presence of CoASH and ATP (Figure 5B and 5C) and the independently measured LCAS reaction (Figure 7) of 3.8-fold or greater increases to 37-fold for the comparison involving estimates of *in vivo* FFA transfer pools. This may in turn be interpreted as 37-fold (or greater, as we are discussing upper limits) concentrated localization of FFA in the export pool *in vivo* (Figure 4) compared to freely diffusing condition (Figure 7). We believe that this leaf tissue to chloroplast comparison is valid

because (1) the chloroplasts are synthesizing fatty acids at rates corresponding to *in vivo* rates of fatty acid synthesis, (2) because the major products from chloroplast assays in the presence of ATP and CoASH are acyl-CoA or acyl-CoA-derived products, (3) because higher maximum velocity and lower FFA affinity for LACS activity are measured with intact organelles loaded with FFA *in situ* rather than with vesicles challenged with exogenous FFA, and (4) because the $t_{1/2}$ value to establish the steady state in isolated chloroplasts could easily be an order of magnitude or more lower if rapid kinetic techniques were available for organelles.

Possible Mechanisms for Fatty Acid Export

The analysis of our data presented above allow us to discount the simple free diffusion-based model for FFA export from the plastid, as shown in Figure 3. There are three generic models (and combinations thereof) to replace the simple diffusion model, namely (1) intervening transfer proteins or (2) a facilitated diffusion process that would mediate the export of fatty acyl groups between the stromal acyl-ACP thioesterase and the outer envelope long chain acyl-CoA synthase, or even (3) a cryptic inner envelope LACS. These models [i.e. for pool (A) in the intact chloroplast assays] are briefly discussed.

For facilitated diffusion without any intervening protein FFA must be deposited in the envelope leaflet where it is most readily used by the LACS enzyme. The limiting *in vivo* FFA transfer pool size of one second calculates to about 0.1 to 0.01 mole% FFA if deposited in a single envelope leaflet lipid phase. In this leaflet the LACS enzyme has a

very high affinity for FFA (low K_m), so that very little FFA has time to move elsewhere.

When the LACS reaction is made inoperative in isolated chloroplasts by lack of CoASH substrate then FFA diffusion to other chloroplast membranes creates pool (B). Little is known about the actual localization, topography and/or vectorial nature of acyl-ACP thioesterases and long-chain acyl-CoA synthetases. Plastid fatty acid synthesis is likely a channeled process (Roughan and Ohlrogge, 1996) associated *in vivo* with envelope membranes as has been observed for acetyl-CoA carboxylase (Thelen and Ohlrogge, 2002) and as suggested by association of acyl ACP thioesterase with membrane fractions when imported into the pea chloroplast by *in vitro* reconstitution experiments (Unpublished observation communicated by Dr. John Froehlich, MSU-DOE Plant Research Laboratory). There are at least nine LACS genes in Arabidopsis (Shockey et al., 2002). One of them, LACS9 (At1g77590), is demonstrated to be localized to the envelope of the plastid (it is not shown whether LACS9 is located on the outer or inner envelope membrane) and was shown to be responsible for the major *in vitro* LACS activity on the plastid envelope (Schnurr et al., 2002). This gene product may therefore belong to the class responsible for the outer envelope LACS activity described previously (Andrews and Keegstra, 1983; Block et al., 1983). To have facilitated FFA diffusion without any additional polypeptides it seems necessary to propose that the fatty acyl ACP thioesterase binds to the inner envelope, possibly causing a localized membrane perturbation which can facilitate FFA flip-flop and loads the hydrolyzed product directly onto the inner envelope. Also, the outer envelope long-chain acyl-CoA synthetase would have a domain that would extend across periplasmic space and interact with the inner envelope in a way that would facilitate FFA transfer.

Conceptually it is easier to envisage a protein-mediated channel (Figure 3). There is evidence from bacterial, yeast and mammalian systems that pairs of proteins are often involved in the vectorial import of fatty acids across biomembranes. In *E. coli* an outer envelope localized protein (FadL) was shown to be necessary for the saturable binding and permeation of exogenous long chain fatty acids (Mangroo and Gerber GE, 1992; Kumar and Black, 1993; DiRusso and Black, 1999). The corresponding acyl-CoA synthetase resides in the inner envelope. In yeast both the FATP protein and the fatty acyl-CoA synthetases of the plasma membrane are interacting components of the fatty acid import machinery (Zou et al., 2003). In animal cells FATP and acyl-CoA synthetases, both plasma membrane proteins, are required for optimum fatty acid uptake (Schaffer and Lodish, 1994). FATP is an AMP-binding domain protein with homology to the very long chain acyl-CoA synthases (Coe et al., 1999). By contrast, activated long chain fatty acid import across the peroxisomal membrane in yeast is mediated by an ATP binding cassette (ABC) transporter (Hettema et al., 1996; Hettema and Tabak, 2000). The data we have obtained are consistent with such an intermediate protein between the acyl-ACP thioesterase and LACS. It is apparent that in the absence of the cofactors for the LACS reaction, nascent FFA (pool (A)) is released (to become pool (B)). This release may appear rapid on the time scale of the assays, but could be quite slow relative to the time taken to channel FFA across the plastid envelope to the LACS protein *in vivo*. If there is only one LACS enzyme then the transport system would also have a finite rate of loading from non-channeled FFA to be able to independently function with freely diffusing FFA. Possible gene candidates for fatty acid transporters might include

At4g14070, At3g23790 and the ABC transporters associated with the plastid envelope (Koo and Ohlrogge, 2002).

The final generic model proposes a latent inner envelope LACS enzyme. Although LACS activity was localized to the outer envelope membrane (Andrews and Keegstra, 1983; Block et al., 1983) we can not definitively rule out the possibility of an inner envelope LACS that is intimately linked to FFA export from the inner leaflet of the inner envelope via an acyl-ACP thioesterase complex, but whose activity is cryptic in a simple LACS assay. Among the possible Arabidopsis gene candidates for "cryptic" LACS might be LACS8 (At2g04350) (Schnurr et al., 2002) or At4g14070 and At3g23790 (AMP-binding proteins with predicted plastid leader sequences). Disruption of a transporter or cryptic LACS might result in an increased FFA pool *in vivo*, and if it has a time constant for removal of the order of seconds, analogous to the removal of pool (B) in chloroplast assays by the LACS dark reaction, then the rapid *in vivo* labeling technique might be useful for the discovery of Arabidopsis lines mutated in these gene products.

In conclusion, we believe that this study defines a set of parameters needed to more fully understand the molecular mechanism of free fatty acid transfer from the plastid. There are several possibilities, as described above, and, given the absence of physical-chemical data on FFA transfer steps for galactolipid-rich membranes and the complexity introduced by the double membrane envelope of plastids, these will not be easy experimental systems. However, the possibility of using reverse genetics in Arabidopsis

should open up tractable new avenues to combine with the traditional ways of studying such problems.

MATERIALS AND METHODS

Materials

Pea seeds (*Pisum sativum* L. cv Little marvel) were germinated on moist vermiculite. For chloroplast preparations the peas were grown for 8-10 days in the growth chamber at 25°C and an 8 h photoperiod with occasional watering. For the *in vivo* experiments the peas were grown for 12 days in the growth chamber watering with nutrient solution. Young pea leaves were removed that had yet to open. These were cut in half laterally, immediately placed in assay medium and preincubated as described below. [1-^{14}C]Acetic acid (57.2 Ci/mol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO).

In vivo Incubations

Leaf assays with halved pea leaves were run in 5.0 ml medium containing 25 mM NaMES buffer, pH 5.7, with 0.3-0.4 g fresh weight of tissue, at 28°C and under strong illumination (180 μmol photons/m^2/s). Tween-20 was added as a wetting agent to a concentration of 0.01% w/v. The reaction was initiated with sodium [1-^{14}C]acetate

solution. Time course assays were run with 0.03-0.06 mCi of labeled acetate (0.105-0.21 mM). These assays were terminated by partial removal of the medium and quenching with hot isopropanol while the tissue was under illumination.

Chloroplast Preparation and Assays

Intact chloroplasts were isolated on a continuous Percoll gradient (Bruce et al., 1994). All procedures were conducted at 0-4 °C. About 10-20g of 8-day old pea seedlings were homogenized in 100 ml of semi-frozen homogenization buffer (50 mM HEPES, pH 8.0, 330 mM sorbitol, 0.1% BSA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA) using a polytron (PT 10/35) and filtered through 2 layers of miracloth (CALBIOCHEM). Crude chloroplasts were collected by centrifugation at 1200 ×g max for 5 min. These were purified by centrifugation through pre-made gradients of Percoll generated by centrifugation of 30 ml of 50% Percoll in homogenization buffer for 30 min at 40,000×g. Crude chloroplasts (2-3 ml) were centrifuged through the pre-made gradients at 13,000 ×g max for 5 min in an HB-4 rotor without brake. The intact chloroplast band formed near the bottom of the gradient was recovered and washed with resuspension buffer containing 50 mM HEPES, pH 8.0 and 330 mM sorbitol. The resulting isolated chloroplasts can incorporate acetate into fatty acids at rates corresponding to *in vivo* rates of fatty acid synthesis, import and process *in vitro* translated protein precursors, sustain high rates of photosynthesis (Keegstra et al., 1986), and give about 90% intactness as determined by phase contrast light microscopy and the reduction of ferricyanide before

and after an osmotic shock (Lilley et al., 1975; Douce and Joyard, 1982). Quantification of chlorophyll was performed as described by Arnon (1949).

Chloroplast incubations to synthesize fatty acids were performed largely according to the methods described by Roughan (Roughan, 1987). Chloroplasts (40-50 μg chlorophyll) were incubated under illumination (180 μmol photons/m^2/s) with shaking at 25°C in a medium (200 μl) containing 0.33 M sorbitol, 25 mM HEPES-NaOH, pH 8.0, 10 mM $KHCO_3$, 2 mM Na_3EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, and 2 mM acetate containing 2 μCi of [^{14}C]acetate. Where noted in the text assays also contained 1 mM ATP, 0.5 mM CoA, and/or 0.5 mg/ml BSA added at specific times. An aliquot was removed before the illumination and used to measure the actual radioactivity added. Reactions were terminated by placing the incubation mixture into liquid nitrogen and were kept in -80°C freezer until lipid analysis.

In the BSA:FFA binding assays, chloroplasts were separated from the BSA in the medium by centrifugation at 13,000 rpm (Sorvall Biofuge pico) for 15 s. The supernatant was aspirated from the chloroplast pellet and frozen in liquid nitrogen. Fatty acids were extracted from the supernatant and the chloroplast pellet separately.

Lipid Analysis

After quenching the tissue by heating in isopropanol at 80-90 °C for 5 min lipid extraction from the [^{14}C]acetate fed leaf strips was carried out using hexane-isopropanol (Hara and Radin, 1978). An aliquot of the lipid extract was suspended in 4:1 acetone/water and the OD at 652nm measured to determine chlorophyll (Arnon, 1949) another aliquot was assayed by liquid scintillation counting to determine radioactivity incorporated. Suspension of the lipid residue in organic solvent and re-evaporation in the presence of a few drops of glacial acetic acid was crucial to drive off residual acetate substrate contamination to give accurate radioactivity determinations. Lipid classes and fatty acid methyl esters were analyzed by thin layer chromatography (TLC) and isolated by preparative TLC. TLC plates were scanned for radioactivity using a Packard Instant Imager (Canberra Instruments), both to quantify radioactivity and to locate the appropriate bands for recovery from preparative TLC plates. For pea leaf extracts a non-saponifable band (4-6% of total label) co-migrated very close to FFA on TLC, so the samples were first treated with ethereal diazomethane and then fatty acid methyl esters determined to measure the free fatty acid pool. Neutral lipids were analyzed on silica plates developed half way with hexane/diethyl ether/acetic acid 70:30:1 (v/v/v) and then fully with hexane/diethyl ether/acetic acid 180:20:1 (v/v/v). For analysis of individual labeled fatty acids the methyl ester fraction was isolated by preparative TLC and separated using either C18-reversed phase TLC, developing half way and then fully with acetonitrile/methanol/water 130:70:1 (v/v/v), or by argentation-TLC using plates impregnated with a 5% silver nitrate in acetonitrile solution, dried and activated, and developed with hexane-diethyl ether mixtures.

The method for analysis of radiolabeled products from chloroplast assays was adapted from Roughan (Roughan, 1987). Samples were saponified by heating in 80-90°C for 60 min in 0.8 ml of 10% KOH (w/v) in methanol per 0.2ml reaction mixture. After cooling, acidification and extraction with hexane and then evaporation of the hexane under nitrogen the total radioactivity recovered was determined by liquid scintillation counting. Since there are negligible non-saponifiable products this radioactivity is a measure of the incorporation of [^{14}C]acetate into total fatty acids. Lipid class analysis was performed by mixing 0.2 ml of reaction mixture with 2 ml of chloroform/methanol 1:1 (v/v) and 0.72 ml of 0.2M H_3PO_4 in 2 M KCl. The lower organic phase was collected and the upper aqueous methanol phase was back-extracted with hexane (4 ml). The combined organic extracts were evaporated to dryness and an aliquot assayed for radioactivity by liquid scintillation counting. Neutral and polar lipids were separated by TLC using K6 silica plates (Whatman, Clifton, PA) developed in hexane/diethyl ether/glacial acetic acid 80/20/1 (v/v/v). To determine the polar lipid composition, the polar lipid band was recovered and analyzed on K6 silica TLC plates impregnated with 0.15 M ammonium sulphate and activated at 120 °C for 3 h, and developed in acetone/toluene/water 91/30/8 (v/v/v) (Kahn and Williams, 1977). The aqueous methanol phase contains acyl-CoA as the major saponifiable lipid. Over 80% of the label was shown to co-migrate with long chain acyl-CoA standard when extracted by butan-1-ol and analyzed by TLC (K6 plate developed in butan-1-ol/acetic acid/water 50:20:25 (v/v/v)). Routinely the aqueous phase was saponified, acidified, extracted with hexane and the extractable lipids (i.e. free fatty acids) assayed for radioactivity (11, 32). Control extractions with chloroplasts spiked

with known amounts of radiolabeled oleic acid or oleoyl-CoA yielded essentially complete recovery of oleic acid and > 85% recovery of oleoyl-CoA either in the presence or absence of BSA.

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CHAPTER 3

SITES AND MECHANISMS FOR ACTIVATION AND ELONGATION OF EXOGENOUS FATTY ACIDS

ABSTRACT

Plant cells are capable of incorporating, elongating and desaturating exogenously provided short- and medium-chain free fatty acids (FFA's). However the subcellular sites and mechanisms for these processes are not currently known. When detached Arabidopsis leaves or isolated pea chloroplasts were incubated with [1-^{14}C]FFA's (≤16-carbon), label appeared in more elongated and desaturated species of fatty acids (FA's). The rate of laurate elongation was 4.9 nmoles /h/mg chlorophyll with intact leaves and 22 nmoles/h/mg chlorophyll with isolated chloroplasts. The elongation by isolated chloroplasts was light dependent, stimulated 1.4 fold by 1mM ATP and was independent of exogenous coenzyme A. The elongation was 60 % inhibited by 10 μM cerulenin, an inhibitor of plastidial ketoacyl-ACP synthetase I/II (KAS I/II). In contrast, haloxyfop, an inhibitor of cytosolic acetyl-CoA carboxylase, inhibited only elongation into very-long chain FA's (≥ C20) but not the synthesis of ^{14}C-unsaturated C18 or C16 FA's. These data indicate that the elongation of exogenous medium-chain FA's into C16 and C18 FA's by Arabidopsis leaves is mainly the function of chloroplasts, most likely through the common *de novo* FA synthesis machineries. The measured pool size of acyl-ACP during [1-^{14}C] laurate elongation by pea chloroplasts was 2.3 μM. In contrast, there was

less than 0.1 nM (or < 1/1000 acyl-ACP concentration) of labeled acyl-CoA indicating that almost no detectable acyl-CoA intermediate was formed during the elongation process. A T-DNA insertion mutant, for a gene (*AAE15*) that is predicted to encode an acyl activating enzyme showed 80% reduction of [1-^{14}C]laurate elongation into C16 and C18 FA's. *AAE15* has sequence similarity to long-chain acyl-CoA synthetases (LACS's) and is predicted to be targeted to the plastid.

INTRODUCTION

It has been observed that plant tissues are capable of incorporating exogenously provided fatty acids into their endogenous lipids. Depending on their length and level of unsaturation, the exogenous fatty acids undergo different metabolic fates. Exogenous [1-^{14}C]palmitic and stearic acid go through the eukaryotic pathway and initially are incorporated into cytosolic glycerolipids mainly at the sn-1 position of phosphatidylcholine (PC) and sn-1 and sn-3 of triacylglycerol (TAG). [1-^{14}C]oleic acid metabolism is similar except that it has less positional preference between sn-1 and sn-2 (Roughan et al., 1987). On the other hand short and medium chain fatty acids of chain length up to 12 carbons have been generally considered to serve as substrates for fatty acid synthesis (Hitchcock and Nichols, 1971). Labeled acetic, octanoic, decanoic, dodecanoic, tetra decanoic acted as precursors for oleic, linoleic and linolenic acids by isolated leaves of *Ricinus communis*, cabbage, lettuce, Brussels sprouts and chicory (James, 1962). Barley seedlings and leaf slices of spinach converted 8 - 14 carbon free fatty acid precursors into longer chain fatty acids (Hawke and Stumpf, 1965; Kannangara CG et al., 1973). Furthermore labeled acetate and laurate were elongated, desaturated and assembled into both prokaryotic and eukaryotic molecular species in Arabidopsis leaves (Norman and St. John, 1986).

Although these elongations of substrates shorter than 16-carbons (C16) were suggested to occur via fatty acid synthesis (FAS) enzymes in the plastid, direct evidence for this

suggestion has not been presented. Nor is the mechanism of how these substrates get activated prior to their involvement in condensation reactions known. *De novo* FAS in plants involves condensation reactions between malonyl-ACP and growing chain of acyl-ACP. Mattoo et al (1989) identified an *in vivo* acylated protein following incubation with exogenous radiolabeled palmitate as acyl-carrier protein in *Spirodela oligorrhiza*. They hypothesized that the exogenous palmitate was first activated to an acyl-CoA derivative and then was transesterified to form acyl-ACP by a side reaction of the condensing enzyme 3-ketoacyl-ACP synthetase (KAS). In their study cell-free extracts from *Spirodela oligorrhiza* formed [^{14}C]palmitoyl-ACP when incubated with [^{14}C]palmitoyl-CoA and ACP. Cerulenin an inhibitor of fatty acid condensing enzyme 3-ketoacyl-ACP synthetase I/II (KASI/II) inhibited *in vivo* formation of acyl-ACP from palmitate but only partially inhibited the formation of acyl-ACP from acyl-CoA in the cell free extract. However, even if this is true with other higher plants, activating exogenous fatty acid into CoA esters in the stroma where transacylation and elongation presumably occurs remains questionable because to date there are no enzymes described that catalyze this reaction.

In this paper we characterize in more detail the elongation of exogenous fatty acids by *Arabidopsis* leaves and isolated pea chloroplasts. Inhibitors of plastid fatty acid elongation and cytosolic malonyl-CoA formation were used to provide information on the subcellular sites of elongation. We have then used this system to test antisense and knock out plants in candidate genes for acyl activating enzymes. These experiments have allowed us to identify an Arabidopsis gene product that is likely involved in activation of exogenous fatty acids in the chloroplast.

RESULTS

Exogenous [1-^{14}C]FFA metabolism by Arabidopsis leaves

When detached leaves of Arabidopsis were incubated with ^{14}C labeled FFA's in the light, the label appeared in essentially all classes of glycerolipids in 3 hours. Table 3 presents the distribution of radioactivity among the major glycerolipids after 3 hours of incubation with short to long chain fatty acid substrates. In 3 h of incubation, shorter fatty acid substrates ($\leq$ 10 carbons) were incorporated preferentially into plastidial lipids (monogalatosyldiacylglycerol (MGDG) ~24%, phosphatidylglycerol (PG) ~10%) whereas longer substrates ($\geq$ 14-carbons) were greatly preferred by cytosolic lipids (PC ~30%). Lauric acid showed an intermediate level of distribution between MGDG (14%) and PC (22%). The distribution of label with acetate indicates that over the 3 h incubation time labeling did not reach equilibrium in order to reflect *in vivo* glycerolipid compositions of Arabidopsis leaves. A previous study showed that when palmitic and stearic acids were fed to spinach leaves they followed eukaryotic pathway being incorporated into PC at first and subsequently were transferred into plastidial galactolipids (Roughan et al., 1987).

Figure 11. displays the time course labeling pattern with [1-^{14}C]laurate. The label initially went rapidly into PC and TAG. The incorporation into PC, PG and MGDG increased approximately linearly until 90 min. In contrast, the rapid incorporation into

Table 3. Distribution of radioactivity among the major glycerolipids following 3 hours of incubation of Arabidopsis leaves with various [1-^{14}C] substrates

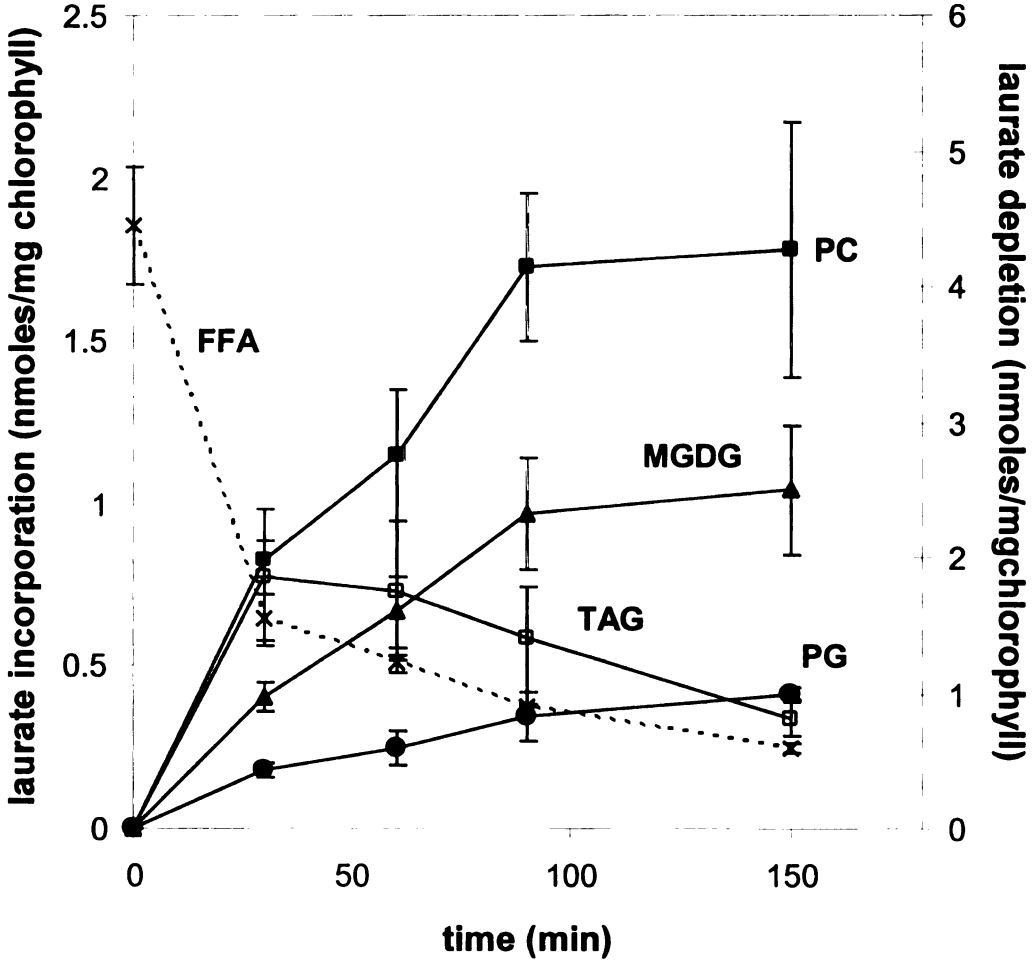
	Exogenous [1-^{14}C] FFA substrates					
	2:0	8:0	10:0	12:0	14:0	16:0
	Percent total radioactivity in the lipid extract (%)					
TAG	[1]-	-	-	2.9	3.7	1.1
FFA	5.2	8.2	5	6.4	9	10
DAG	1	-	1.3	2.3	2.9	3.2
MGDG	23.9	24	25.6	14.2	5.6	2.8
PG	10.4	10.8	9.8	5.8	4.5	3.9
DGDG	1.9	2.5	2.7	1.8	1.2	1.2
PI,PS,SL	2.4	2.6	3.2	3.3	3.6	4.2
PE	1.9	2	2.7	3.6	4	6.1
PC	14.4	10.4	16.8	22.2	31.4	31.4
Sum	61.1	61.9	67.3	62.5	65.9	63.9

Note. Detached Arabidopsis leaves were incubated in 25mM NaMES (pH5.7) buffer containing [1-^{14}C] labeled substrates indicated in the table under illumination at 25°C for 3 hours.

[1] < 1%.

Figure 11. Time course incorporation of [1-^{14}C]laurate into glycerolipids by detached Arabidopsis leaf.

Detached Arabidopsis leaves (20-30 days old) were incubated in 25mM NaMES (pH5.7) buffer containing 10 μM [1-^{14}C]laurate under illumination at 25°C for 2.5 h. Samples were removed from light and were frozen at designated time points. Averages of 3 different extractions are plotted with error bars (±SD).



TAG ended by 30 min after which label in TAG decreased presumably through transfer into other lipids or to degradation.

Analysis of total lipid extracts by transmethylation revealed the elongation and desaturation pattern of each fatty acid substrates (Table 4). In all cases, label was found in fatty acids longer than the substrates but there was an inverse relationship between substrate chain length and efficiency for use in elongation and desaturation. With ≤ C10 FA substrates over 95% of the radioactivity in the fatty acid extract was found to be elongated or desaturated after 3 h of incubation. For [1-^{14}C]lauric acid and [1-^{14}C] myristic acid 65% and 37% of the label in the lipid extract was elongated respectively. Palmitate was the least suitable substrate for elongation (13%) and especially for desaturation. Reduced desaturation in the labeled products was also observed as the length of substrates used grew longer. The elongation reaction with little desaturation might suggest direct elongation of the MCFA substrates by extraplastidial FA elongase system some of which might be involved in wax synthesis in the epidermal cells.

The rate of exogenous laurate incorporation into longer fatty acids calculated at 20 min is about 4.9 nmoles C12/h/mg chlorophyll (Figure 12A). This translates to about 11 nmoles C2/h/mg chlorophyll (C14 20%, C16 50%, C18 28%, C20 0.64%, C22 0.64%, C24 1.3%, C26 0.64%). For comparison, with 10μM acetate the incorporation rates into fatty acid measured in spinach leaf and isolated spinach chloroplast are about 10–100 nmoles/h/mg chlorophyll (Roughan et al., 1978; Pollard and Ohlrogge, 1999). Although it is difficult to make direct comparison between incorporation of different substrates especially when

Table 4. Distribution of radioactivity among the total fatty acids following 3 hours of incubation of Arabidopsis leaves with various [1-^{14}C] substrates

	Exogenous [1-^{14}C] substrates					
	2:0	8:0	10:0	12:0	14:0	16:0
FAME classes	Percent total radioactivity in the lipid extract (%)					
10:0	-[2]	-	1.3	-	-	-
12:0	0.9	1.0	0.6	32.3	-	1.3
14:0	0.6	1.3	1.7	8.2	61.1	0.9
16:0	24.4	30.3	33.2	25.5	22.7	75.1
16:1	2.7	3.7	3.0	1.8	0.8	0.2
16:2	3.4	5.0	3.8	2.5	0.8	0.3
16:3	2.6	4.6	4.7	2.8	1.1	-
18:0	3.9	3.1	3.3	2.5	2.7	6.8
18:1	28.7	19.4	20.3	10.0	3.1	1.1
18:2	13.7	11.2	11.3	5.8	1.9	0.3
18:3	9.0	8.4	11.5	4.9	1.7	-
≥ 20:0	3.8	5.9	4.3	2.1	2.6	6.3
Sum of elongated products[1]	94.0	93.4	97.3	66.4	37.2	13.8

Note. Detached Arabidopsis leaves were incubated in 25mM NaMES (pH5.7) buffer containing [1-^{14}C] labeled substrates indicated in the table under illumination at 25°C for 3 hours. At the end of the incubation, buffers were removed and leaves were washed with water and blotted briefly on filter paper. The leaves were frozen with liquid nitrogen and stored in -80°C until lipid extraction.

[1] percentage of label in the fatty acids longer than applied substrates.

[2] < 0.2%.

they have markedly different uptake efficiencies, solubility, enzyme specificities and cellular toxicity effects, nevertheless, the laurate elongation rate seems to be similar or slightly lower than fatty acid synthesis rate from acetate.

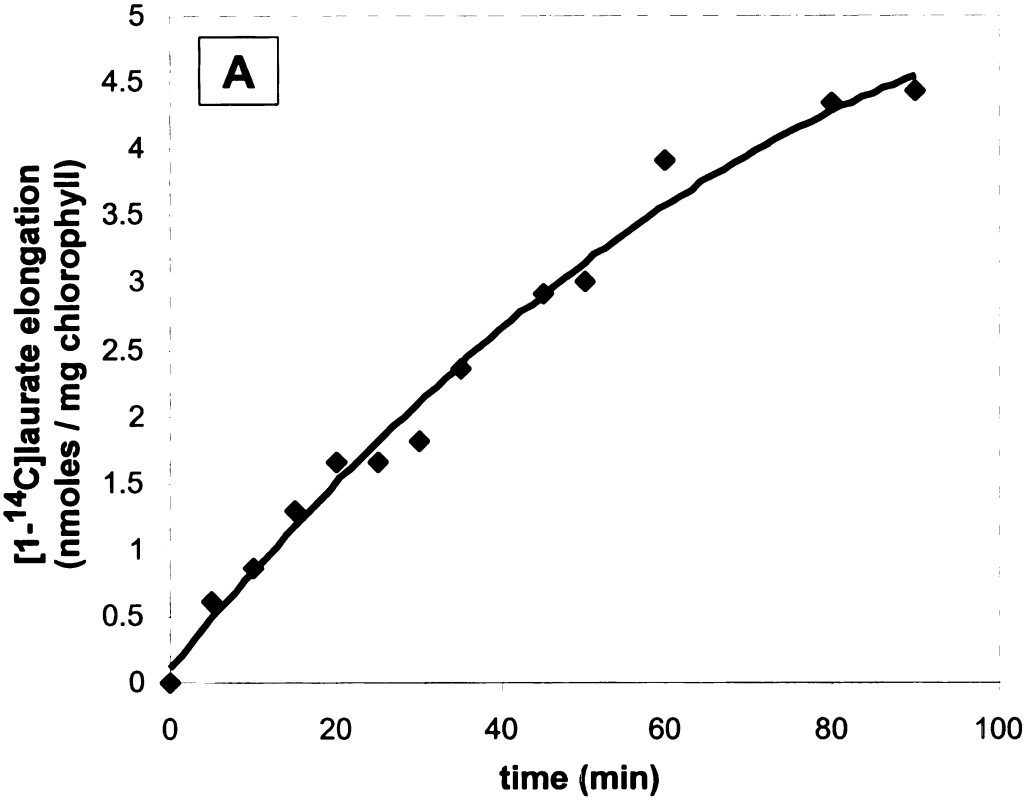
The time course of elongation of [1-^{14}C]laurate into individual FA's (Figure 12B, minor components are not shown) showed that the highest proportion of labeled fatty acid species were palmitate followed by oleate and myristate. The steady state synthesis rates of 14:0, 16:0, 16:1, 18:0 and 18:1 were maintained for about 30 min or less whereas more elongated and desaturated species steadily increased until 90 min or more. Although there was still laurate in the lipid extract at the 30 min time point, it is possible that availability of substrates for elongation became limiting by 30 min. The more elongated ($\geq$ C20 FA's) and desaturated species (16:2/16:3, 18:2/18:3) continued to accumulate at the expense of shorter and less desaturated precursors.

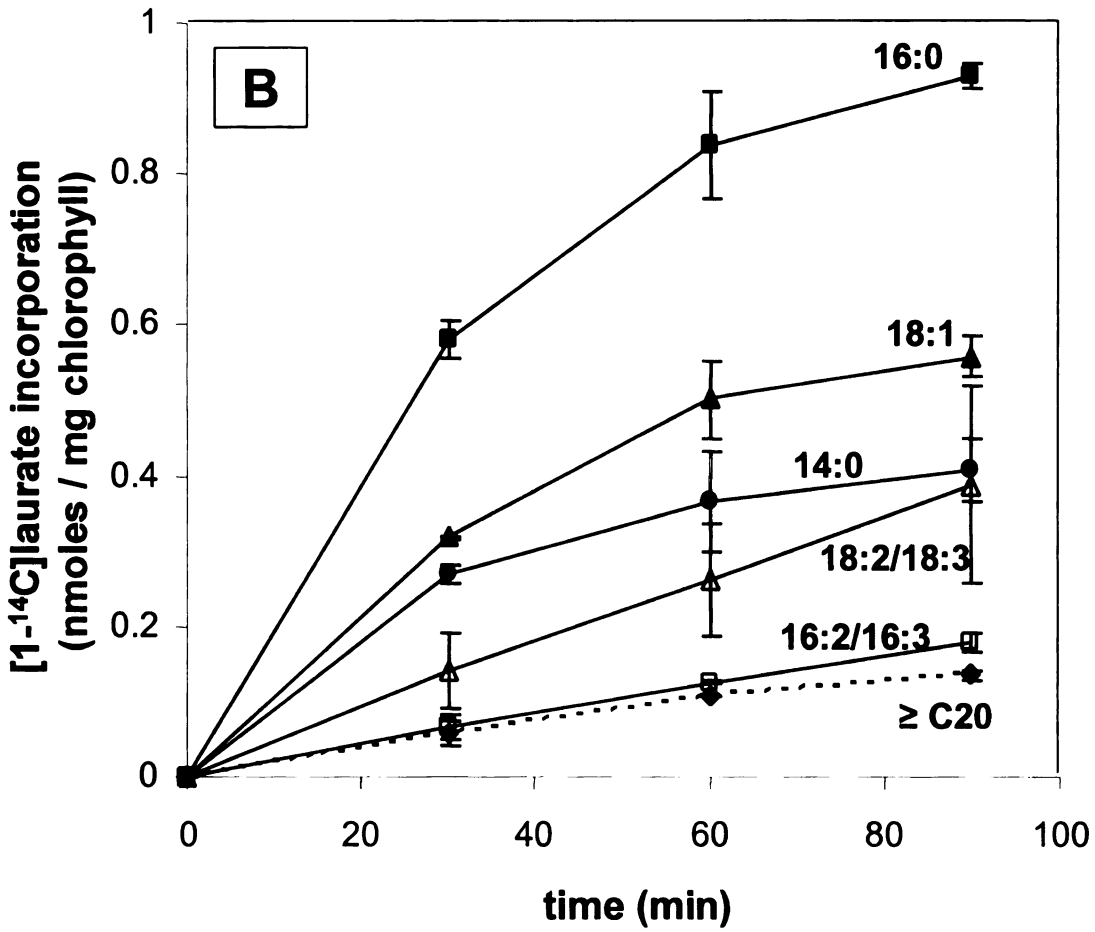
Effect of plastidial FAS inhibition on exogenous laurate elongation by leaves

It was previously assumed that the elongation of exogenously fed short and medium chain fatty acids takes place in the chloroplast where *de novo* fatty acid synthesis takes place (Hitchcock and Nichols, 1971; Norman and John, 1986; Thompson Jr. et al., 1986; Roughan et al., 1987). However, no direct evidence for this assumption has been presented and a mechanism to activate fatty acids to ACP in the chloroplast has not been described. Plants also have the ability to elongated FA's by a membrane bound ER system which might provide an alternative to plastids. In order to investigate the

Figure 12. Time course elongation of [1-^{14}C]laurate substrate by detached Arabidopsis leaf.

Detached Arabidopsis leaves (20-30 days old) were incubated in 25mM NaMES (pH5.7) buffer containing 10μM [1-^{14}C]laurate under illumination at 25°C for 90 min. Samples were withdrawn from light at various time points and individual fatty acids were analyzed by TLC following transmethylation (see Material and Method). A. Overall [1-^{14}C]laurate incorporations into > C12 FA's are plotted. B. Elongation into individual FA's is shown. Averages of 3 different extractions are plotted with error bars (±SD).


A
[1-14C]laurate elongation
(nmoles / mg chlorophyll)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
time (min)


B
16:0
18:1
14:0
18:2/18:3
16:2/16:3
≥ C20
[1-14C]laurate incorporation
(nmoles / mg chlorophyll)
time (min)
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80
100

cytosolic contribution to laurate elongation, cerulenin, an inhibitor of plastidial KASI/II enzymes, was used. If the cytosolic contribution is major then we would expect laurate to be elongated despite inhibition of plastidial fatty acid synthesis.

Detached Arabidopsis leaves were incubated with either [1-^{14}C]acetate or [1-^{14}C]laurate under illumination for 1 h in the presence of various concentrations of cerulenin. Figure 13A displays the effect of cerulenin concentration on laurate elongation by Arabidopsis leaves. [1-^{14}C]acetate incorporation into fatty acids was used as control of effectiveness of cerulenin. Acetate incorporation was inhibited about 85% at 50μM of cerulenin and 95% at 200μM. Laurate elongation was inhibited with an almost identical dose dependence. Two conclusions can be made from this experiment. First, the major activity of laurate elongation into C16 and C18 FA's is most likely associated with KAS I/II and is localized to the plastid. Second, laurate apparently is a poor substrate for direct elongation by cytosolic fatty acid elongation enzymes. Figure 13B shows cerulenin effect on laurate incorporation into glycerolipids. Cerulenin inhibition of elongation had almost no effect on laurate incorporation into PC and TAG. Since laurate was not able to elongate in the presence of cerulenin, incorporation into PC and TAG most likely was mediated by acyl transferases that had activity on laurate as substrates. On the other hand label appearance in MGDG, PG and DGDG was greatly reduced by the inhibition of elongation.

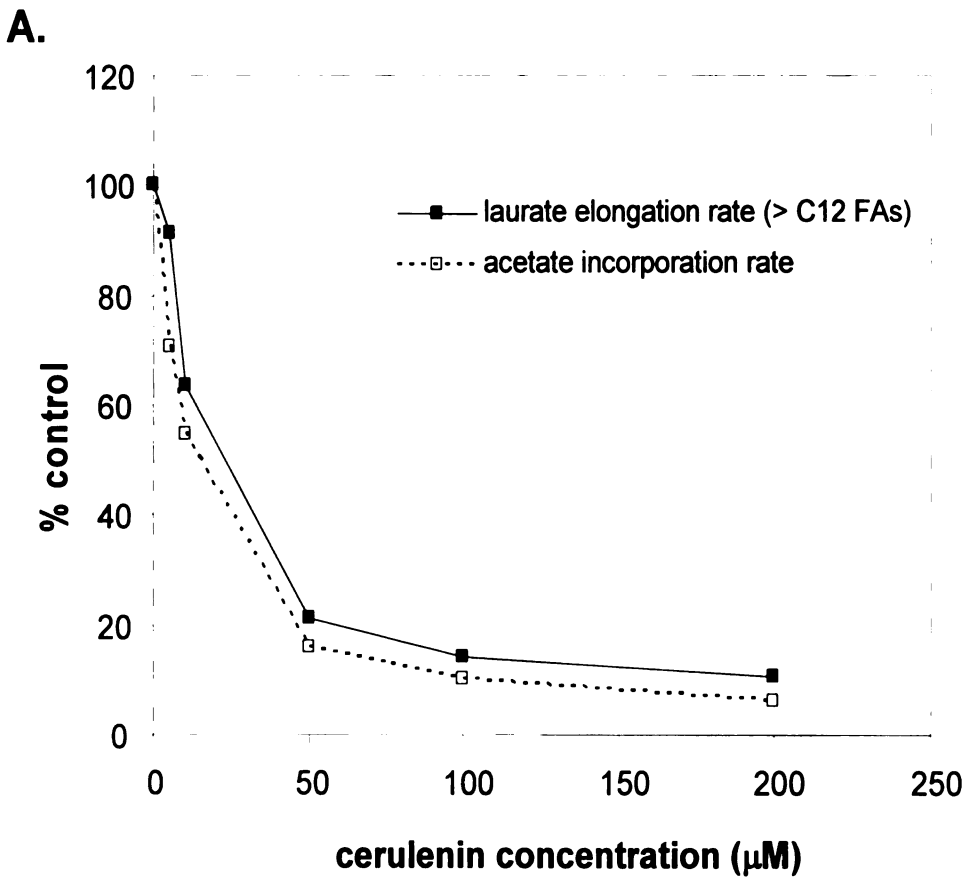
Effect of cytosolic FA elongation inhibition on exogenous laurate elongation by detached Arabidopsis leaves

Acetyl-CoA carboxylase (ACCase) catalyzes activation of CO_2 and its transfer to acetyl-CoA to form malonyl-CoA, the carbon donor of fatty acid synthesis. There are 2 types of ACCase found in dicots with different sensitivity to herbicides such as haloxyfop. The heteromeric ACCase in plastids that provides malonyl-CoA for *de novo* FAS is insensitive, whereas the cytosolic homodimeric ACCase, that supplies malonyl-CoA for fatty acid elongation is inhibited by haloxyfop (Bao et al, 1998).

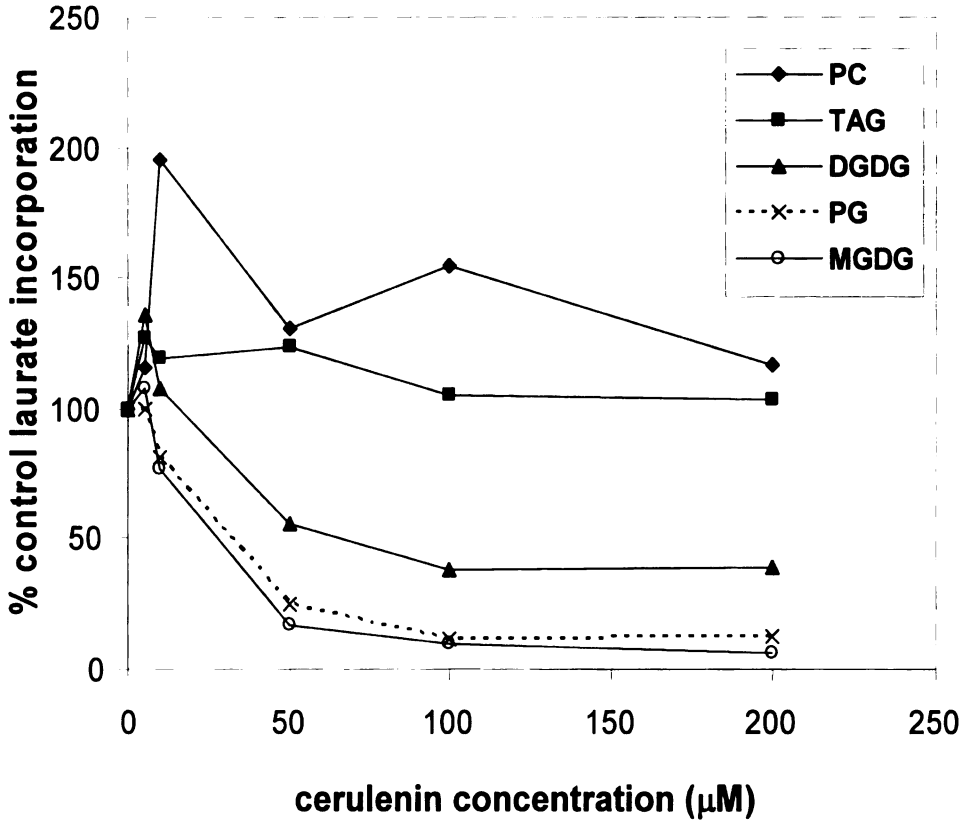
When detached leaves of Arabidopsis were incubated with [1-^{14}C]laurate and increasing amounts of haloxyfop, elongation into $\geq$ C20 FA's was selectively inhibited (60-70%) whereas there was almost no effect on overall synthesis of FA's up to C18 (Figure 14). These data demonstrate that the initial elongation of laurate into palmitic and oleic acid is not inhibited by haloxyfop and supports the conclusion from the cerulenin experiments that this process occurs mainly in plastids. However, synthesis of 14:0 and 18:0 was moderately reduced by haloxyfop. An interpretation consistent with this result is that a portion of stearate synthesis from exogenous laurate occurs after palmitate is exported from plastids. Furthermore, these data suggest that part of the 14:0 FA products are derived from a haloxyfop sensitive entity, either cytosol or mitochondria.

Figure 13. Cerulenin effect on elongation of [1-^{14}C]laurate by detached Arabidopsis leaves.

Detached Arabidopsis leaves were incubated with either [1-^{14}C]acetate or [1-^{14}C]laurate as described previously with increasing concentrations of cerulenin. A. Cerulenin effect on acetate incorporation into FA's and laurate elongation. B. Cerulenin effect on [1-^{14}C]laurate incorporation into major glycerolipids.



B.


250
200
150
100
50
0
% control laurate incorporation
0
50
100
150
200
250
cerulenin concentration (μM)
PC
TAG
DGDG
PG
MGDG

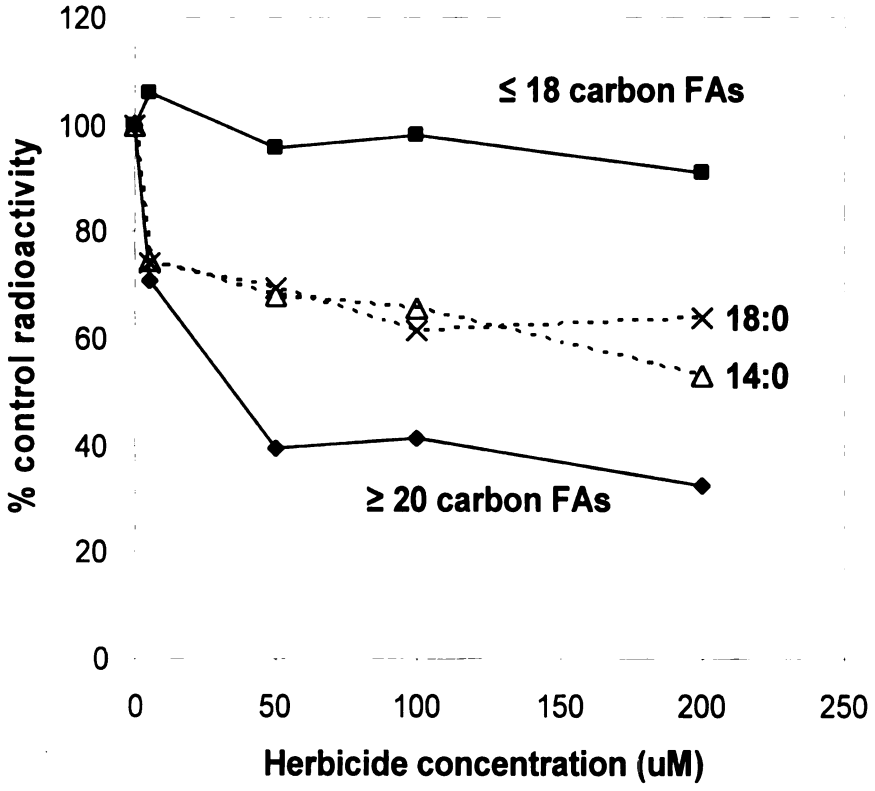
Contribution of recycled acetate units on the labeled products of exogenous FA elongation by detached Arabidopsis leaves is small

The interpretation of the labeling patterns presented so far could be confused if the added [1-^{14}C]fatty acids were subject to β-oxidation and ^{14}C products were recycled into *de novo* FAS. To evaluate this possibility we determined the distribution of ^{14}C in the oleic acid product after [1-^{14}C]-fatty acid and acetate labeling. Oxidative cleavage at the double bond position of oleic acid methyl ester creates nonanoic acid (C9A) and 1,ω-nonane-dioic monomethyl ester fragments (C9AE). If ^{14}C in oleic is derived from [1-^{14}C]-acetate, the theoretical distribution of label between the two 9 carbon fragments is 0.8. If ^{14}C in oleic is derived only from intact [1-^{14}C] FA precursors and there is no contribution of recycled acetate units, either C9AE or C9A will be labeled depending on the length of precursor used.

Table 5 presents the distribution of label among the two oxidative fragments of ^{14}C-labeled oleic acid after Arabidopsis leaves were incubated with different [1-^{14}C]FA substrates. ^{14}C-oleic acid from leaves labeled with [1-^{14}C] acetate showed close to the theoretical (0.8) C9A/C9AE ratio of 0.9. When [1-^{14}C] labeled C10 through C14 FA's

Figure 14. Haloxyfop effect on elongation of [1-^{14}C]laurate by detached Arabidopsis leaf.

Detached Arabidopsis leaves were incubated with [1-^{14}C]laurate as described previously with various concentrations of cerulenin for 30 min under illumination. Samples were withdrawn from light at various time points and individual fatty acids were analyzed by TLC following transmethylation.



were used as substrates almost all ^{14}C label was recovered in the carboxylic half of oleic acid indicating recycled ^{14}C-acetate was not a major contributor of label in the oleic acid. [1-^{14}C]Octanoic acid substrate labeled mostly the methyl end of oleic acid (83%). These results are consistent with an early study made with spinach leaf slices labeled with 6:0, 8:0, 10:0, 12:0 FA's that showed little β-oxidative degradation and re-synthesis when examined by reductive ozonolysis of the C18 unsaturated fatty acids (Kannanagara et al., 1973).

Exogenous laurate elongation by isolated pea chloroplasts

Isolated pea chloroplasts (40μg chlorophyll) were incubated under illumination for 20 min with 10μM [1-^{14}C]laurate (0.2 μCi). Figure 15A displays the time course of [1-^{14}C]laurate incorporation into elongated FA's. The overall rate of elongation into > C12 FA determined at the first 10 min was 22 nmoles C12/h/mg chlorophyll. This is about 4 times faster than that was measured in the intact leaf. The difference may reflect difficulties for exogenous laurate to penetrate the leaf cuticle and leaf cells to reach the plastid, cytotoxicity of unesterified laurate, competition by endogenous substrates, or perhaps feedback regulation in the intact leaf compared to isolated chloroplasts.

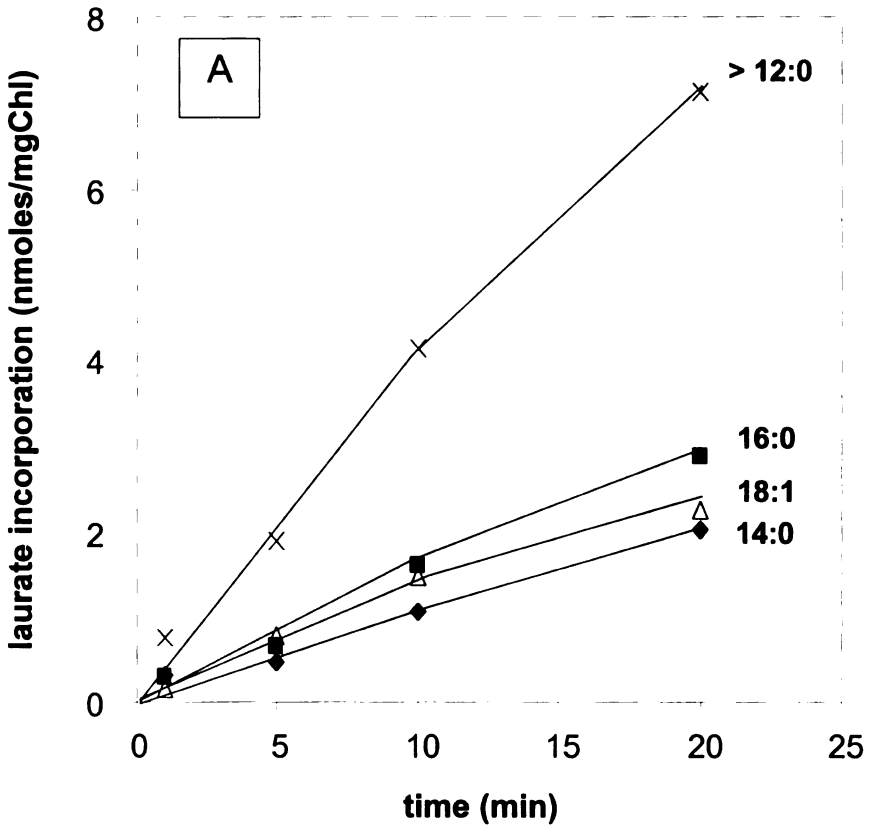
Table 5. Label distribution among the oxidative cleavage fragments of ^{14}C-labeled oleic acid of Arabidopsis leaves incubated with different [1-^{14}C] labeled FA substrates.

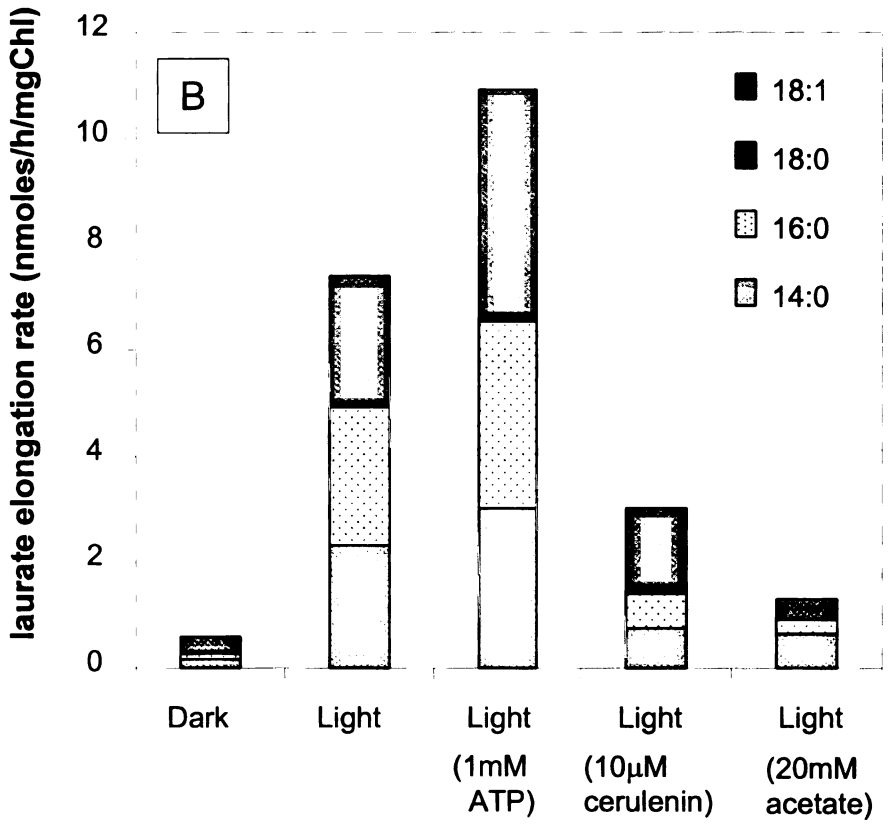
[1-^{14}C] substrates	2:0	8:0	10:0	12:0	14:0
% total radioactivity in each oxidative cleavage fragments					
C9A	37.2	82.8	4.4	3.4	1.6
C9AE	40.4	14.5	96.4	95.2	88.4
ratio between the oxidative cleavage fragments					
C9A / C9AE	0.9	5.7	0.04	0.04	0.02

Note. Oleic FAME was recovered and cleaved at the double bond by permanganate-periodate oxidation (Christie, 2003). The resulting nonanoic acid (C9A) and 1,ω-nonane-dioic monomethyl ester fragments (C9AE) were examined by TLC.

Figure 15. Elongation of exogenous [1-^{14}C]laurate by isolated pea chloroplasts.

A. Time course of [1-^{14}C]laurate elongation. Isolated pea chloroplasts (40μg chlorophyll) were incubated under illumination in a medium described in the 'Material and Methods' section with 1mM ATP and 10μM [1-^{14}C]laurate (0.2 μCi). Each data point represents average value of 2 independent lipid extractions and analyses. B. Characteristics of [1-^{14}C]laurate elongation. Isolated pea chloroplasts (80μg chlorophyll) were incubated either in the presence or absence of light with 10μM [1-^{14}C]laurate (0.2 μCi) as substrate for 20 min. Indicated amounts of ATP, cerulenin, unlabeled acetate were included in the media. Mean of 3 different extractions were used to plot the graph.


A
> 12:0
16:0
18:1
14:0
laurate incorporation (nmoles/mgChl)
time (min)
0
2
4
6
8
0
5
10
15
20
25


laurate elongation rate (nmoles/h/mgChl)
12
10
8
6
4
2
0
B
18:1
18:0
16:0
14:0
Dark
Light
Light (1mM ATP)
Light (10μM cerulenin)
Light (20mM acetate)

Several characteristics of laurate elongation by isolated pea chloroplasts are shown in Figure 15B. No detectable elongation was observed in the dark. Although the elongation was not dependent on exogenously provided ATP, 1mM ATP stimulated the elongation about 1.4 fold in the light as observed also for fatty acid synthesis from acetate (Koo et al., 2004). Low concentrations (1-2 mM) of unlabeled acetate increased laurate incorporation into elongated FA's (data not shown). However 20 mM unlabeled acetate inhibited the incorporation of exogenous [1-^{14}C]laurate into elongated FA's to less than 18% of control (without unlabeled acetate). The elongation of [1-^{14}C]laurate was sensitive to cerulenin and was inhibited in a manner that is similar to the inhibition of [1-^{14}C]acetate incorporation into fatty acids by cerulenin. Overall elongation of laurate was reduced 60% with 10μM cerulenin (Figure 15B) and almost completely with 50μM cerulenin (data not shown). These experiments with isolated chloroplast indicate that the chloroplast has the ability to elongate exogenous MCFA through the *de novo* FAS system.

How do exogenous FA's get activated in the plastid?

The data presented so far indicate that the majority of the exogenous MCFA elongation activities are associated with plastid *de novo* FAS. But how do the exogenous FA's get activated prior to their use in FAS in the plastid? The existence of a pathway in plants for esterification of exogenous FA to ACP has only been inferred in past studies (Kannangara et al., 1973; Terzaghi, 1986; Thompson Jr. et al., 1987). Mattoo et al.

(1989) identified an acylated soluble protein as ACP following the incubation of Spriodela plant culture with radiolabeled palmitate. They hypothesized that exogenous FA was first activated to the acyl-CoA derivative and subsequently transesterified to ACP via a side reaction of the FAS condensing enzyme, (Alberts et al., 1972). In support of this hypothesis cell free extracts from *Spirodela* were able to catalyze the formation of palmitoyl-ACP from palmitoyl-CoA whereas the cell free system could not esterify free palmitic acid to ACP. Even if we suppose this idea is true we still need either an enzyme to catalyze formation of long chain acyl-CoA in the stroma / inner membrane of plastid where FAS occurs or a transport system to introduce long chain acyl-CoA formed at the cytosolic side of plastid double membrane into the site of FAS. An alternative possibility might be direct formation of acyl-ACP from free exogenous FA's in the stroma / inner membrane. Acyl-ACP synthetase (EC 6.2.1.20) has been isolated from bacteria and yeast (Rock and Cronan Jr., 1981; Fice et al., 1993; Jackowski et al., 1994; Gangar et al., 2001). Lessire and Cassagne (1979) also reported the direct synthesis of stearoyl-ACP from stearate in a microsomal fraction from *Allium porrum* epidermal cells although at a much slower rate compared to acyl-CoA formation (about 1%).

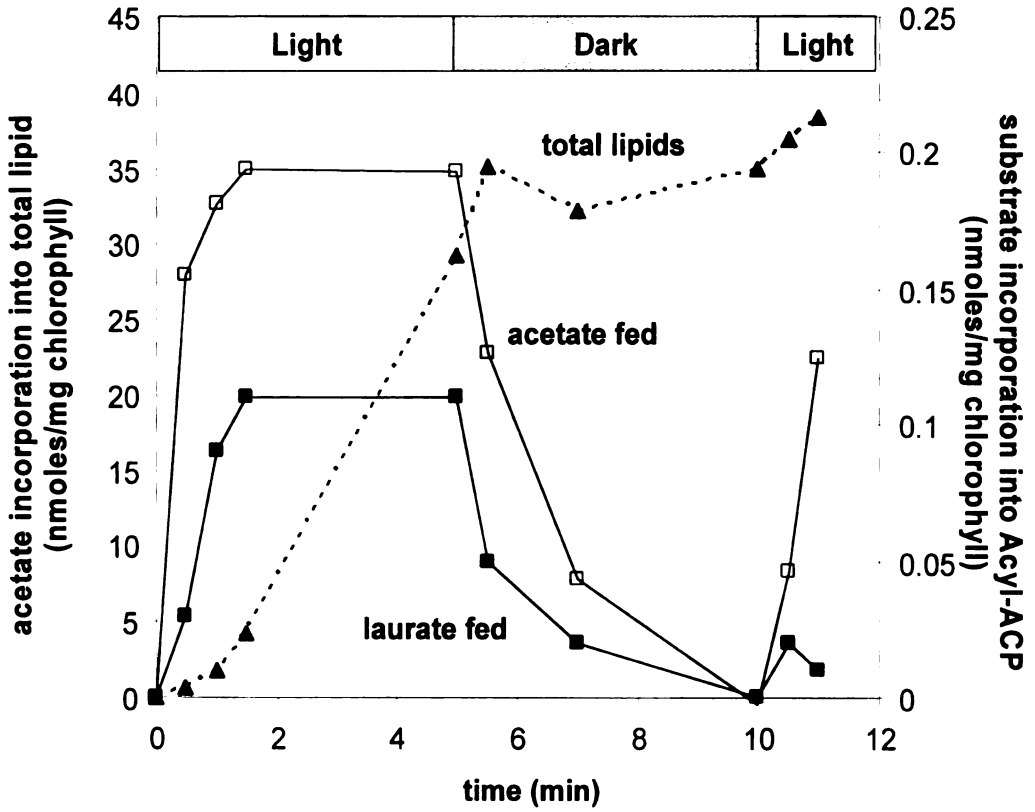
We used two different approaches to understand the activation process of exogenous FFA in the plastid. First we examined acyl-ACP and acyl-CoA pools formed during the elongation of exogenous [1-^{14}C]laurate in the isolated pea chloroplasts. Second, we tested several Arabidopsis mutants/transgenics disrupted in gene candidates that might be involved in the acyl activation processes.

Intermediates of exogenous [1-^{14}C]laurate elongation in isolated pea chloroplasts

The procedure of Mancha et al. (1975) was used to isolate the acyl-ACP fraction from pea chloroplasts incubated with [1-^{14}C]laurate or [1-^{14}C]acetate. Lipids were first removed by extraction with organic solvent and proteins were subsequently precipitated in the presence of ammonium sulfate and chloroform / methanol. The acyl esters in the pellets were then transmethylated and separated from any FFA contaminants by TLC (Mancha et al., 1975; Soll and Roughan, 1982). In order to verify that the recovered acyl-ACP fraction did contain acyl-ACP molecules, the time course labeling pattern of acyl-ACP fractions in the light and in the dark were examined as shown in Figure 16. Acetate incorporation into total lipid increased linearly in the light. Upon removal of light, acetate incorporation stopped immediately and resumed with re-illumination. The labeling pattern of acyl-ACP fraction with both acetate and laurate showed typical labeling pattern of intermediates with high turn over rates. Within about 1 min of labeling in the light, acyl-ACP fraction reached saturation and did not increase further in the light. In the dark, label disappeared rapidly and completely. The estimated pool size of [1-^{14}C]acyl-ACP was 0.5-2.3μM assuming a chloroplast volume of 47 μL/mg chlorophyll (Wirtz et al., 1980). The reported total ACP molecule content in spinach chloroplast was 8μM (Ohlrogge et al., 1979) and total acyl-ACP in the acetate labeled spinach chloroplast was estimated at 5μM (Soll and Roughan, 1982).

Figure 16. Time course light synthesis and dark catabolism of acyl-ACP by isolated pea chloroplasts.

Isolated pea chloroplasts (90μg chlorophyll) were incubated under illumination and in the dark in a basal medium (Materials and Methods) with either 0.2mM [1-^{14}C]acetate (5 μCi) or 13 μM [1-^{14}C]laurate (0.36 μCi) as substrates. Total lipids as well as acyl-ACP's were extracted following direct quench at each time points. Acetate incorporation into total lipid (▲) follows left axis scale. [1-^{14}C]Acetate (□) and [1-^{14}C]laurate (■) incorporation into acyl-ACP fractions are depicted following right axis scale.



To detect radiolabeled acyl-CoA in these incubations, several different extraction methods were employed. We tested (i) direct separation of the reaction mixture by TLC (butanol/acetic acid/water developing system) following rapid quench and solubilization with chloroform/methanol (1/1), (ii) a modified version of acyl-CoA separation procedure developed by Mancha et al.(1975), and (iii) butanol extraction of aqueous phase after lipid extraction (Bligh and Dyer,1959) followed by TLC separation. With each method, recovery of [1-^{14}C]-acyl-CoA standards was at least 60%, but we could detect less than 0.1 nM (or < 1/1000 of acyl-ACP concentration) of labeled acyl-CoA in the isolated pea chloroplasts labeled with [1-^{14}C]laurate. These results indicate that, in contrast to acyl-ACP formation, almost no detectable acyl-CoA intermediate was formed during the elongation process.

Laurate elongation activity in Arabidopsis mutants disrupted in expression of candidate genes for plastid acyl activation

To further investigate the activation of laurate to ACP by chloroplasts we took a bioinformatics approach to select potential Arabidopsis genes that might be involved in MCFA activation in the plastid (Table 6). A superfamily containing 63 different genes encoding acyl-activating enzymes (*AAE*) in Arabidopsis genome has been described (Shockey et al., 2003). Among those were 3 members of acetyl-CoA synthetases (*ACS's*) and 11 members of long chain acyl-CoA synthetases (*LACS*'s). *LACS*'s were characterized in detail previously for their ability to complement a LACS-deficient strain

of yeast and for the *in vitro* LACS enzyme activity in the cell free lysates of yeast or *E. coli* cells overexpressing the candidate *LACS* genes (Shockey et al., 2002). Nine of these candidates were found to have LACS enzyme activity. One of those 9 members, *LACS9* (At1g77590), was demonstrated to be localized to the envelope of the plastid (it is not known whether *LACS9* is located on the outer or inner envelope membrane) and was shown to be responsible for the major *in vitro* LACS activity on the plastid envelope (Schnurr et al., 2002).

Because laurate elongation was associated mainly with plastids, we selected candidates that were reported to be plastidial or had plastid targeting sequence as predicted by Target P (Emanuelsson et al., 2000, http://www.cbs.dtu.dk/services/TargetP/). One of above 3 *ACS*'s and 4 of 11 *LACS*'s had predicted plastidial targeting sequences with reliability classes ranging from 1 through 5 (Table 6). At5g36880 was reported to be a plastidial *ACS* in a previous study (Behal et al., 2002). *In vitro* chloroplast import assays of *LACS8* (At2g04350) in the studies by Schnurr et al (2002) was reported to be positive. *AAE15* (At4g14070) and *AAE16* (At3g23790) had sequence elements leading to categorization as *LACS's* along with other 9 *LACS* members before. The predicted proteins have a high reliability class for plastidial targeting. Shockey et al. (2002) reported that both *AAE15* and *AAE16* could not complement the mutant phenotype of a yeast mutant deficient in *LACS* (C14-C18 FA's were tested) and neither had activity in *in vitro* assays using cell-free lysates of yeast expressing *AAE15* and *AAE16* and using oleic acid as substrate. Overexpression strains of *E. coli* also lacked LACS activity for 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3 and 20:1 FA substrates (Shockey et al., 2002). In a study of fatty acid

export from plastids, Koo et al. (2004) raised the possibility of *AAE15* and *AAE16* as "cryptic" LACS's that are perhaps linked to FFA substrates coming out from the stroma upon release from acyl-ACP thioesterase.

To test the possible involvement of these candidates in exogenous FFA activation in the plastid we took a reverse genetics approach. The idea is to test the ability of Arabidopsis mutants impaired in those selected gene candidates to elongate exogenous [1-^{14}C]laurate substrate. *ACS* gene silenced lines were kindly sent to us for test by Dr. David J. Oliver from Iowa State University (Behal et al., 2002). These T2 seeds had about 15% to 30% of wildtype *ACS* activity (Oliver DJ, personal communication). T-DNA insertion mutant lines for *LACS8*, *AAE15* and *AAE16* were searched from the Sequence-Indexed Library of Insertion Mutations generated by the Salk Institute Genome Analysis Laboratory using Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). Homozygous KO lines were selected among the T3 generations by PCR screening for both the presence of T-DNA insertion and the absence of an intact gene. The lines tested for [1-^{14}C]laurate elongation activity were T4 generation homozygous knock-outs. The insertion was at the 8th intron for LACS8 KO (*lacs8*, ABRC stock identifier SALK_ 136060) and at the 7th intron and 2nd exon respectively for *AAE15* KO (*aae15*, SALK_ 139663) and *AAE16* KO lines (*aae16*, SALK_067859).

Table 6. Summary of gene candidates of exogenous FFA activation in the plastid tested in this study.

gene	MIPS description	Locus	[1] subcelluar localization	[2] TM domain	mutant lines
ACS	Acetyl CoA synthetase	At5g36880	C (5)	1	Antisense
LACS8	Putative acyl-CoA synthetase	At2g04350	C (5)	1	SALK_136060
AAE15	AMP-binding protein	At4g14070	C (2)	none	SALK_139663
AAE16	AMP-binding protein, putative	At3g23790	C (1)	none	SALK_067859

[1] Target P (web server at http://www.cbs.dtu.dk/services/TargetP/) was used to predict subcelluar localization. Reliability class (RC) values are shown in parenthesis.

[2] TMHMM v2.0 (web server at http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used for α helical transmembrane domain prediction.

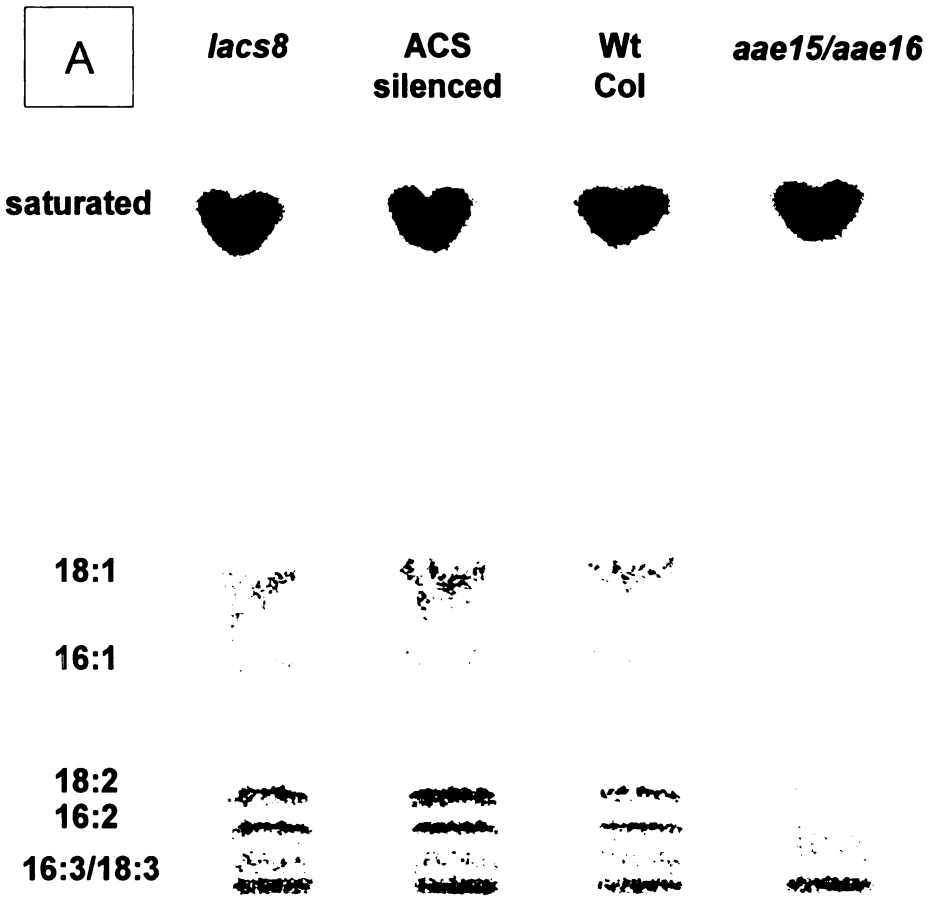
Detached leaves from *ACS* gene silenced lines, *lacs8, aae15/aae16* and wild type (ecotype Columbia) Arabidopsis were incubated for 20 min with [1-^{14}C]laurate. Displayed in Figure 17 are an image of radiolabeled FAME classes separated on silver nitrate impregnated TLC plate (A) and a corresponding graph presents the laurate elongation rates (B). Bands corresponding to 18:1, 16:1, 18:2, 16:2 and 18:3/16:3 (orders from top to bottom) are greatly reduced in *aae15/aae16*. There was about 80% reduction in overall elongation of laurate into both C16 and C18 FA's. No significant changes were observed with *ACS* antisensed lines and *lacs8*.

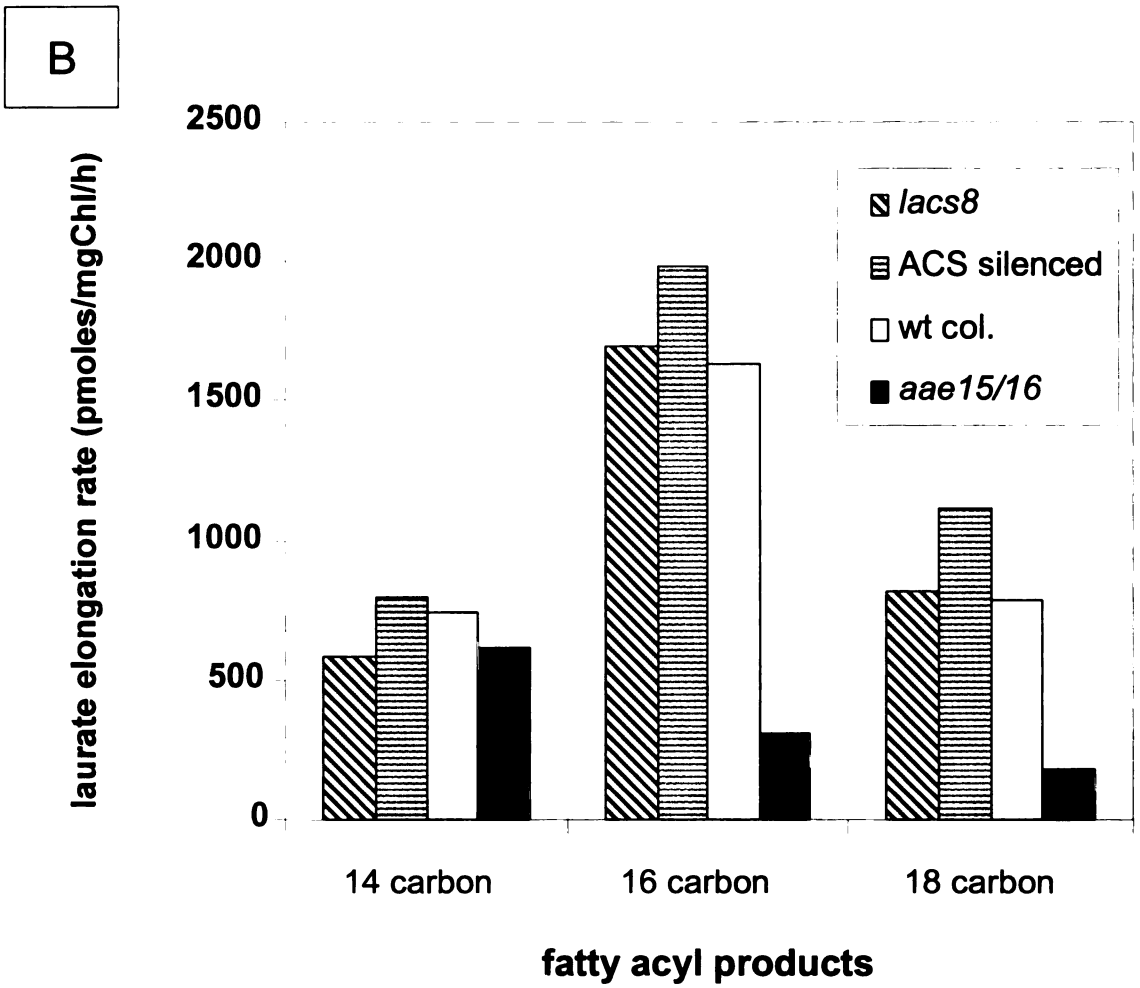
Elongation into 14:0 FA was apparently not affected. It has been noted previously (Figure 13A and Figure 14) with inhibitors of cytosolic and plastidial FAS that at least some portion of the elongation into 14:0 was contributed by both haloxyfop sensitive (i.e. ER, mitochondrial) and cerulenin sensitive (i.e. plastid, mitochondrial) entities. The fact that elongation into 14:0 FA was not blocked in the plant that were knocked out in elongation activity of exogenous laurate into C16 and C18 FA might suggest that when plastidial elongation pathway gets blocked cells may redirect the flux through ER or mitochondrial pathways.

In order to find out which gene among *AAE15* and *AAE16* was responsible for the defect in exogenous MCFA elongation activity, single knock-outs were tested for elongation activity (Figure 18). Since it is also possible that there could be substrate specificities among the two gene products, we tried 4 different substrates, [1-^{14}C]labeled 2:0, 8:0,

Figure 17. Exogenous [1-^{14}C]laurate elongation by wild type and mutant lines of candidates for plastidial acyl activating enzymes.

Detached leaves of each lines were incubated in 25mM NaMES (pH5.7) buffer containing indicated [1-^{14}C]FFA substrates under illumination for 20 min. A. Image of [1-^{14}C]FAME classes separated on silver nitrate impregnated TLC plate. B. [1-^{14}C]Laurate incorporation rate into major FA's.

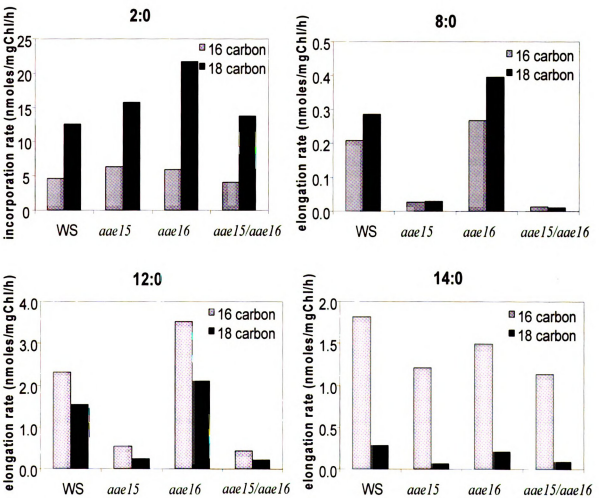



B
2500
2000
1500
1000
500
0
laurate elongation rate (pmoles/mgChl/h)
lacs8
ACS silenced
wt col.
aae15/16
14 carbon
16 carbon
18 carbon
fatty acyl products

12:0 and 14:0. [1-^{14}C]Acetate was used as a control to test whether the disruption of MCFA elongation was due to some defect in FAS machinery other than activation process. There were no differences in acetate incorporation rate into FA's in both single knock-outs (*aae15*, *aae16*) and double knock-out (*aae15/aae16*) compared to wild type (WS). Elongation of [1-^{14}C]8:0 into both C16 and C18 FA's was consistently reduced to 10% or less of wild type elongation rate, in the *aae15* and *aae15/aae16* plants. Elongation activity in *aae15* and *aae15/aae16* was reduced to about 20% wild type with [1-^{14}C]12:0 substrate. Both *aae15* and *aae15/aae16* retained about 60% of elongation activity into C16 FA with [1-^{14}C]14:0 substrate. The observation that elongation was reduced to a lesser degree with longer substrates is consistent with the idea that some portion of elongation activity was coming from extra plastidial origin and that it had preferences for longer substrates. With all substrates tested there was no apparent change in the *aae16* plant. We conclude that disruption of the *AAE15* gene product was responsible for defective elongation of exogenous MCFA by Arabidopsis leaves.

Figure 18. Elongation of [1-^{14}C]acetate and [1-^{14}C]MCFA substrates by detached leaves of wild type and single/double knock outs of *AAE15* and *AAE16*.

Detached leaves of each lines were incubated in 25mM NaMES (pH5.7) buffer containing indicated [1-^{14}C]substrates under illumination for 20 min. Individual fatty acids were analyzed by TLC following extraction and transmethylation.



DISCUSSION

Exogenous [1-^{14}C]FFA metabolism in Arabidopsis leaves

Metabolism of exogenously provided fatty acids by plants has been studied in the past with emphases on their incorporation into endogenous lipids (James, 1962; Hawke and Stumpf, 1965; Kannangara CG et al., 1973; Norman and St. John, 1986; Roughan et al., 1987). The incorporated fatty acyl moieties were often found elongated and desaturated as well. In this study we have tried to dissect further the compartmentation and metabolism of exogenous FA's in Arabidopsis detached leaves with an emphasis on understanding where and how the exogenous MCFA gets elongated. We used Arabidopsis leaves as a model system in order to take advantage of genomic information and mutants that could guide reverse genetic strategies.

Fatty acid molecules that are entering from outside the cells might be expected to partition into virtually all endomembrane systems. However we are focusing on organelles that are known to be involved actively in fatty acid metabolism. The main subcellular fractions to consider in terms of fatty acid metabolism in plant leaves are endoplasmic reticulum, plastids and peroxisomes. In addition biochemical studies of pea leaf mitochondria indicated that mitochondria are capable of *de novo* FAS beginning from malonate (Wada et al., 1997) although the activity was much lower (< 1%) than plastidial FAS.

The endogenous substrates for glycerolipid acyltransferases are mainly 18:1, 16:0 and 18:0. The exogenous FA may either be used directly for acyltransfer reactions or first elongated and then be used. Incorporation of exogenous FA's into PC and TAG preferred longer chain ($\geq$ 14 carbons) FA substrates whereas shorter fatty acid substrates ($\leq$ 10 carbons) were preferentially incorporated into the plastidial lipids (Table 3). It may appear that plastidial glycerolipids accepted shorter substrates as their substrates. However in reality it was the opposite. The time course labeling pattern of lipids by exogenous [1-^{14}C]laurate (Figure 11) indicated that the direct use of exogenous laurate in PC and TAG synthesis was considerable. Moreover this reaction was insensitive to cerulenin inhibition of *de novo* FAS (Figure 13B). On the other hand laurate incorporation into MGDG, DGDG and PG were severely affected by cerulenin treatment suggesting they must be elongated to appropriate chain lengths prior to their further metabolism by the plastid. The poor incorporation of 14:0 and 16:0 into plasitidial lipids may be due to either a preference by cytosolic enzymes, resulting in these fatty acids being sequestered before they reach the plastid or alternatively their poor usage in the plastid elongation reactions. Over 95% of the radioactivity in the fatty acid extract was found to be elongated after 3 h of incubation with $\leq$ C10 FA substrates (Table 4). The percent elongation decreased inversely with an increase in chain length with $\geq$ C12 FA's. In addition when palmitate was fed to isolated pea chloroplasts, there was no obvious synthesis of oleic acid after 30 min although most of the palmitate was in free available form (data not shown).

The TAG content of leaves is usually minimal (<1% of lipid). However, when leaves were fed exogenous laurate, over 15% of label appeared in TAG at 30 min. This incorporation was transient and after 30 min, the label in TAG started to decrease while incorporation into other glycerolipids continued. This redistribution does not seem to be due to the depletion of FFA substrates because about 25% of the total label was still recovered as FFA at 30 min and the decreased label in the TAG can not account for the increased label in PC, MGDG and other major glycerolipids which continued over 90 min. Rapid incorporation of exogenous FFA into TAG and transfer to other destination could be general response of cells to deal with excess FFA's in leaves.

The plastid is the major site of exogenous MCFA elongation into C16 and C18 FA's

Stearoyl ACP desaturases (EC1.14.19.2) which introduces the first double bond in 18:0-ACP reside in the chloroplast. Desaturation of palmitate at **Δ**3 and **Δ**7 in higher plants also occurs in the chloroplasts (Browse et al., 1985; Kunst et al., 1989). Thus the level of desaturation may serve as a marker for exogenous FA entry into the chloroplast. The saturated to unsaturated ratio of ^{14}C products formed from [1-^{14}C]octanoic and decanoic acid treated samples were slightly higher (0.8) than with [1-^{14}C]acetate (0.6). This ratio increased 5 fold with [1-^{14}C]myristate and up to 16 fold with [1-^{14}C]palmitate (Table 4). James (1962) also observed that although palmitate and stearate were incorporated into leaf lipids they were not able to act as precursors for oleic, linoleic and linolenic acids in leaves of *Ricinus Communis* after 3 to 5 h incubation. However desaturation of 16:0 and

18:0 into 16:3 and 18:3 was observed in spinach leaves with longer incubation time (> 8 h) (Thompson Jr. et al., 1986; Roughan et al., 1987). Although it was not shown explicitly that this was not through incorporation of acetate unit generated by β-oxidation, the authors argued that there being minimal amount of labeled C16 fatty acids in leaves treated with [^{14}C]stearate is indirect evidence that contribution by FA recycling was small (Roughan et al., 1987). Elongation of exogenous FA's with little desaturation might suggest direct elongation of the MCFA substrates by extraplastidial FA elongase system some of which might be involved in wax synthesis in the epidermal cells (Kunst and Samuels, 2003).

Isolated pea chloroplasts were able to elongate [1-^{14}C]laurate into myristic, palmitic, and oleic acids at rates faster than measured with the intact leaves demonstrating their ability to incorporate exogenous MCFA into *de novo* FAS machinery. However, this doesn't exclude the possible contribution of other sources in exogenous MCFA elongation in whole leaf system. We used inhibitors to examine the mechanism and subcellular site of MCFA elongation in the whole leaf. Cytosolic FA elongation requires malonyl-CoA production by eukaryotic ACCase. In addition there is no mitochondrial isoform of ACCase found in Arabidopsis, suggesting it is dependent on cytosolic production of malonate. The eukaryotic homodimeric ACCase is sensitive to several herbicides that have no effect on the multisubunit ACCase (Burton et al., 1987,1991; Ohlrogge and Browse, 1995). On the other hand the 3-ketoacyl-ACP inhibitor, cerulenin is expected to inhibit the plastidial and mitochondrial elongation system, allowing only cytosolic

elongation to operate. About 85% of acetate incorporation into fatty acids by Arabidopsis whole leaf was blocked by 50μM cerulenin and 95% by 200μM cerulenin. Cerulenin was slightly less (5%) effective on exogenous laurate elongation than on acetate incorporation (Figure 13A). Although natural substrates for the cytosolic elongation system are 16:0, 18:1 and 18:0 exported from plastid, our data suggested that 5-10% of the laurate elongation may occur through direct elongation of laurate by a cytosolic elongation system. However over 90% of the elongation activity comes either from plastid or mitochondria. The blockage in the elongation into very long chain fatty acids was a secondary effect of reduced 16:0, 18:0, and 18:1 production. When detached leaves of Arabidopsis were incubated with [1-^{14}C]laurate with increasing amounts of haloxyfop, laurate elongation into very long chain (≥ C20) FA's were selectively inhibited. Laurate elongation into C16, C18 FA's especially into the unsaturated series was increased or not affected. The elongation not being sensitive to haloxyfop provides further evidence that the plastid is the major site of MCFA elongation into C16/C18 FA's.

Perhaps the most critical evidence of the plastid being the major source of MCFA elongation into C16/C18 FA's came from the knock-out plant impaired in a gene predicted to be targeted to the plastid. In *aae15*, elongation activity was reduced down to 10-20% of the wild type. Together these data confirm the plastid as the major source of MCFA elongation into C16/C18 FA's.

A possibility of an additional contribution by mitochondria may be suggested at least with the accumulating pool of 14:0. Myristic acid is usually a minor component in the leaves/isolated chloroplasts incubations with [1-^{14}C]acetate. However, an unusually high portion of label was found in 14:0 FA when [1-^{14}C]laurate was used as substrate (Table 4, Figure 15). This activity was sensitive to KAS inhibition by cerulenin. Unlike unsaturated C16 and C18 FA's, accumulation of 14:0 was affected, although not as much as elongation into very long chain FA's, by the cytosolic ACCase inhibitor, haloxyfop. Since Arabidopsis mitochondria don't possess their own ACCase they are expected to rely on cytosolic production of malonate for FAS and thus thought to be sensitive to haloxyfop treatment. Mitochondrial FAS system is also sensitive to cerulenin (Wada et al., 1997). Laurate elongation into 14:0 was not affected in *AAE15* knock-out plants further supporting a separate origin for the ^{14}C-14:0 accumulating in intact leaves. The 14:0 accumulation in isolated chloroplast might therefore be due to contamination of different fractions in the isolated plastids preparation. It is also possible that FA synthesis system in damaged chloroplast may contribute to certain extent where coupling between elongating products and FAS system is less tight thereby allowing growing chain of intermediates to escape prematurely.

Identification of acyl activating enzyme in the plastid through reverse genetics approach

The above analysis of subcellular sites of elongation does not identify the crucial step needed to activate acyl chains before their elongation. All elongation steps in *de novo* FAS involve condensation between malonyl-ACP and a growing chain of acyl-ACP and therefore exogenous FFA must be activated prior to involvement in any further FAS reactions. Likewise the desaturation of exogenous palmitate and stearate observed in the prolonged incubation of spinach leaves that was mentioned earlier (Thompson Jr. et al., 1986; Roughan et al., 1987), presumably requires activation of free FA substrates in the plastid since the enzymes that introduces the first double bond to palmitoyl ACP or stearoyl ACP resides in the plastid (Browse et al., 1985; Kunst et al., 1989) unless there's palmitoyl-, stearoyl-CoA desaturase in the cytosol. Acyl-CoA synthesis in isolated chloroplasts incubated with [^{14}C]acetate is absolutely dependent on exogenous ATP and coenzyme A as cofactors either in the dark or in the light (Koo et al., 2004). However, exogenous MCFA elongation in isolated chloroplasts is independent of exogenous ATP or coenzyme A (Figure 15B). This implies an activation reaction that is separate from the described LACS activity of long chain fatty acids in chloroplasts. There is no described fatty acyl-activating enzyme in the stroma; neither acyl-CoA synthetase nor acyl-ACP synthetase. The Arabidopsis mutant that had T-DNA insertion in *AAE15* gene was normal in fatty acid synthesis activity when [1-^{14}C]acetate was used as substrate showing that it possessed intact FAS enzymes (Figure 18). However elongation of exogenous [1-^{14}C]MCFA was severely reduced. *AAE15* (At4g14070) gene has similarity to other known *LACS*'s and contains sequence elements characteristic for *LACS*'s including the classical AMP binding domain and additional linker domain

(Shockey et al., 2002) but is distinguished from the other 9 members of *LACS* family because it was not able to complement the mutant phenotype of yeast deficient in *LACS* nor had *LACS* activity in the cell-free lysates of both yeast and *E.coli* over expressing the gene (Shockey et al., 2002).

Both the subcellular localization predictors (both ChloroP and TargetP) and the biochemical data presented in this paper indicate *AAE15* is a plastidial enzyme. *AAE15* was also represented in the list of recently identified Arabidopsis plastid envelope proteins through mass spectrometry based proteomics approach (Ferro et al., 2003). There is a disagreement concerning predicted α helical transmembrane domains between different predictors. TMHMM v2.0 (Krogh et al., 2001) and TopPred 2 (Claros and von Heijne, 1994) did not detect any obvious α helical transmembrane segments whereas TMpred (Hofmann and Stoffel, 1993) and DAS (Cserzo et al., 1997) predicted 1 transmembrane domain region. However, it isn't clear yet exactly how *AAE15* is involved in exogenous FA elongation. As mentioned above elongation in the isolated chloroplast occurs without an extra supply of cofactors. As shown in Figure 16 there are at least two possible ways of converting exogenous FFA into acyl-ACP. One is via forming acyl-CoA intermediate first and then transacylating it into acyl-ACP. Bacterial FAS condensing enzyme was reported to have acyl-CoA:ACP transacylase activity as a side reaction (Alberts et al., 1972). Plastidial KAS might also be able to catalyze this step. Mattoo et al., (1989) reported formation of [^{14}C]palmitoyl-ACP when a cell-free extract of *Spirodela oligorrhiza* was incubated with [^{14}C]palmitoyl-CoA and ACP.

Cerulenin inhibited *in vivo* formation of acyl-ACP from palmitate supporting the idea of KAS involvement in this step but only partially inhibited the formation of acyl-ACP from acyl-CoA in the cell free extract indicating alternative ways to this reaction end. A second possible route of acyl-ACP formation is the direct esterification of free fatty acid with ACP. Acyl-ACP synthetase (EC 6.2.1.20) has been isolated from bacteria and yeast (Rock and Cronan Jr, 1981; Fice et al., 1993; Jackowski et al., 1994; Gangar et al., 2001) but not in plant. Lessire and Cassagne (1979) reported the direct synthesis of stearoyl-ACP from stearate in a microsomal fraction from *Allium porrum* epidermal cells although at a much slower rate compared to acyl-CoA formation (about 1%). Our analysis of intermediates formed during laurated elongation favor the direct formation. When isolated pea chloroplasts were incubated with [1-^{14}C]laurate, and elongation was allowed, rapid formation of [1-^{14}C]acyl-ACP was detected (0.5-2.3 μM)(Figure 16). However, with three methods we failed to find detectable levels of [1-^{14}C]acyl-CoA intermediates. If there were indeed acyl-CoA intermediates the pool size is expected to be at least one thousand fold lower than acyl-ACP concentration in the stroma. However, we cannot rule out a very small pool of acyl-CoA is an intermediate in the formation of the acyl-ACP.

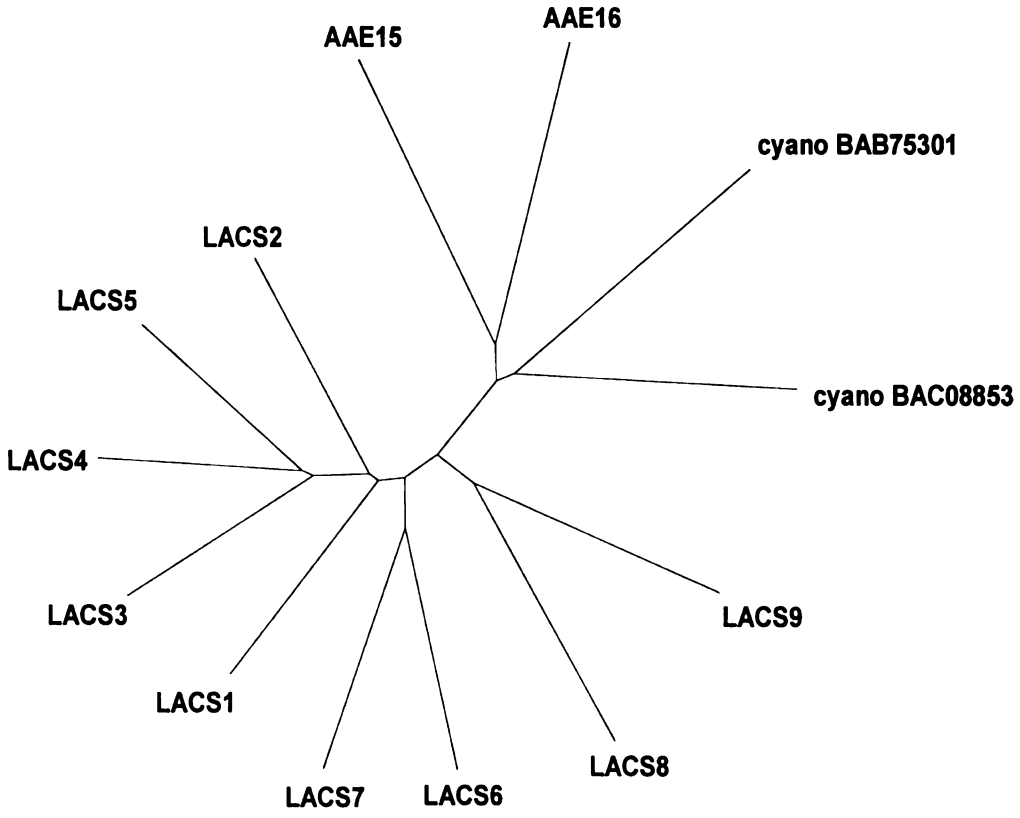
What is the in vivo function of FA activation/elongation in the plastid?

Exogenous fatty acids are important energy sources for unicelluar organisms and animal cells. However intercellular transport and exchange of lipid molecules is minimal among

plant tissues. Thus activation and elongation of exogenous FA may look irrelevant to 'normal' physiological function of plants. Indeed *aae15* does not show any visible phenotypes under normal growth conditions in the growth chamber. However, the gene product of *AAE15* may become important under certain conditions. *AAE15* has 11 EST's in the public EST libraries and thus is expressed at levels similar to many enzymes of lipid metabolism. Based on the reduced elongation of 8:0, 12:0, and 14:0 in *aae15* (Figure 18), the substrate specificity of *AAE15* toward medium chain fatty acids may be broad. This suggests one hypothetical function of *AAE15* might be membrane editing and proof reading of *de novo* FAS. Fatty acid elongation during *de novo* FAS in the plastid halts when the growing chain of fatty acid gets hydrolyzed from ACP by action of fatty acyl-ACP thioesterase. If the shorter fatty acids escape FAS system prematurely and gets released into the membrane, there may need to be mechanisms to recapture them for return to the regular process. Also there could be occasions other than biosynthetic error where potentially harmful free fatty acids can get released from the membrane, including stress conditions and mechanical wounding. In this case the released FFA's are expected to be the long-chain fatty acids which do not need to be elongated. Perhaps *AAE15* may act as both acyl-ACP synthetase and acyltransferase. The *E. coli AAS* gene product was shown to possess 2 enzymatic activities that work in concerted manner; first, to activate exogenous free fatty acid into acyl-ACP and second, to transfer the acyl group to 2-acylglycerophosphoethanolamine to synthesize phosphatidylethanolamine. It is possible that *AAE15* protein recycles the released FFA's and lysoglycerolipids under stress conditions. Nine out of 11 *AAE15* EST's are derived from libraries that were constructed

Figure 19. Phylogenetic comparison of *AAE15* and other *LACS* genes.

The deduced full-length amino acid sequences of the genes that contained both the AMPBP signature motif and the linker domain were aligned by T-Coffee (Notredame et al., 2000, web server at http://www.ch.embnet.org/software/TCoffee.html) and was displayed as an unrooted nearest neighbor phylogenetic tree using the TreeView program (Page et al.,1996).



after dehydration and cold treatments. The expression pattern of *AAE15* in these conditions may give us clue to the *in vivo* function.

One other interesting characteristic of *AAE15* is that it's closest homolog other than *AAE16* and one of Brassica *LACS's* (accession: CAA96521, Fulda et al., 1997) was the long chain fatty acid CoA ligase from cyanobacteria *Nostoc* sp. PCC 7120 (accession: BAB75301) (Figure 19). Their expected location in the chloroplast and their similarity to cyanobacterial *LACS* makes it interesting topic to study in respect to their origin.

MATERIALS AND METHODS

Materials

Wild-type *Arabidopsis thaliana* (ecotype Columbia (Col) was used in all detached leaf incubation experiments unless otherwise indicated as Wassilewskija (WS).

ACS gene silenced lines were kindly provided for us to test by Dr. David J. Oliver from Iowa State University (Behal et al., 2002). T-DNA insertion mutant lines for *LACS8* (At2g04350, seed stock identifier SALK_136060), *AAE15* (At4g14070, SALK_139663) and *AAE16* (At3g23790, SALK_067859) were searched from the Sequence-Indexed Library of Insertion Mutations generated by the Salk Institute Genome Analysis Laboratory using Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) and the seed stocks were ordered from Arabidopsis Biological Resource Center (ABRC). The double knock-out line of *AAE15* and *AAE16* (*aae15/aae16*) was kindly provided by Dr. John Browse (Institute of Biological Chemistry, Washington State

University) and Dr Martin Fulda (Department for Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Sciences, Georg-August-University).

All Arabidopsis plants were grown at 80 to 100 μmol/m^2/s and 22°C under an 18-h-light/6-h-dark photoperiod. Pea seeds (*Pisum sativum* L. cv Little marvel) were grown for 8-10 days in the growth chamber at 25°C and an 8 h photoperiod with occasional watering. [1-^{14}C]Acetic acid (57.2 Ci/mol), [1-^{14}C]ocatanoic acid (55mCi/mmol), [1-^{14}C]decanoic acid (55mCi/mmol), [^{14}C]dodecanoic acid(55mCi/mmol), [1-^{14}C]tetradecanoic acid(55mCi/mmol), [1-^{14}C]hexadecanoic acid(55mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide) was purchased from Sigma and was used as 50mM stock in absolute ethanol. The herbicide haloxyfop was a gift from DowElanco (Indianapolis, IN) and was used as 100mM stock in 100% methanol.

Homozygous T-DNA Insertion Mutant Isolation

T-DNA (5.5-kb) insertions into the *LACS8, AAE15* and *AAE16* genes were identified by PCR screening for both the presence of T-DNA insertion and the absence of intact gene. The gene-specific primers used for the screening of insertions into the *LACS8* gene were 5'-CTGATTCGCAACGCTTCATC-3' (forward) and 5'-CTATAGCTGATGGAAGTGG-3' (reverse), and for *AAE15* were 5'-ATCATCACTGGTCACACAGG-3' (forward) and 5'-AAGGCAGCAATTACTCTCGC-3' (reverse), and for *AAE16* were 5'-

AACCTCTTATCTCCTCAGCC-3' (forward) and 5'-CATCGACAGGAGTTATCAGC-3' (reverse). The T-DNA– specific primer matching the left end of the T-DNA was 5' TGGTTCACGTAGTGGGCCATCG 3'.

Arabidopsis detached leaf incubations

Twenty to thirty days old detached Arabidopsis leaves (0.2g fresh weight/assay) were incubated in 25mM NaMES (pH5.7) buffer containing 0.01% w/v amount of tween-20 as wetting agent under illumination (180 μmol photons/m^2/s) at 25°C for designated length of times. For cerulenin and haloxyfop inhibition assays, the leaves were pre-incubated in above media containing various concentrations of inhibitors for 20 – 30 min. The reactions were initiated by adding 0.1-1 μCi's of [1-^{14}C] labeled fatty acid substrates in 5-10μM concentration (1mM unlabeled acetate was added in case with [1-^{14}C]acetate substrate) as indicated in the Figure legends. At the end of each incubations, buffer was removed and leaves were washed with excessive amount of water and blotted briefly on to the filter paper. The leaves were frozen with liquid nitrogen and stored in -80°C until lipid extraction.

Chloroplast preparations and assays

Intact chloroplasts were isolated on a continuous Percoll gradient (Bruce et al., 1994). All procedures were conducted at 0-4 °C. About 10-20 g of 8-day old pea seedlings were

homogenized in 100 mL of semi-frozen homogenization buffer (50 mM HEPES, pH 8.0, 330 mM sorbitol, 0.1% BSA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA) using a polytron (PT 10/35) and filtered through 2 layers of miracloth (CALBIOCHEM). Crude chloroplasts were collected by centrifugation at 1200 ×g max for 5 min. These were purified by centrifugation through pre-made gradients of Percoll generated by centrifugation of 30 mL of 50% Percoll in homogenization buffer for 30 min at 40,000×g. Crude chloroplasts (2-3 mL) were centrifuged through the pre-made gradients at 13,000 ×g max for 5 min in an HB-4 rotor without brake. The intact chloroplast band formed near the bottom of the gradient was recovered and washed with resuspension buffer containing 50 mM HEPES, pH 8.0 and 330 mM sorbitol. The resulting isolated chloroplasts can incorporate acetate into fatty acids at rates corresponding to *in vivo* rates of fatty acid synthesis, import and process *in vitro* translated protein precursors. Quantification of chlorophyll was performed as described by Arnon (1949).

Chloroplast incubations to synthesize fatty acids were performed largely according to the methods described by Roughan (1987). Chloroplasts (80 μg chlorophyll) were incubated for designated amount of time under illumination (180 μmol photons/m^2/s) with shaking at 25°C in a medium (200 -500μl) containing 0.33 M sorbitol, 25 mM HEPES-NaOH, pH 8.0, 10 mM $KHCO_3$, 2 mM Na_3EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, and with either 0.2mM [1-^{14}C]acetate (5 μCi) or 10 μM [1-^{14}C]laurate (0.1 μCi). Where noted in the text, assays also contained 1 mM ATP, designated concentration of cerulenin and unlabeled sodium acetate. An aliquot was removed before the illumination and used

to measure the actual radioactivity added. Reactions were terminated by placing the incubation mixture into liquid nitrogen and were kept in -80°C freezer until lipid analyses.

Lipid analysis

Total lipids from labeled leaf samples were extracted according to the method of Hara and Radin (1978). After quenching the tissue by heating in isopropanol at 80-90 °C for 5 min lipid extraction was carried out using hexane-isopropanol. An aliquot of the lipid extract was suspended in 4:1 acetone/water and the OD at 652nm measured to determine chlorophyll (Arnon, 1949); another aliquot was assayed by liquid scintillation counting to determine radioactivity incorporated. TAG, FFA, DAG were separated on regular K6 TLC using hexane / diethylether / acetic acid (v/v/v=80/20/1). Other glycerol lipids were separated by developing on ammonium impregnated K6 TLC with acetone: toluene: water (v/v/v=91/30/8). For individual fatty acyl group analysis, lipid extract was first transmethylated by heating at 90°C for 1 h in 0.1 mL of toluene and 1 mL of 10% (v/v) boron trichloride:methanol (Sigma). After acidification with aqueous acetic acid, fatty acid methyl esters were extracted two times with hexane. Fatty acid methyl esters (FAME's) were separated by argentation-TLC using plates impregnated with a 7.5% silver nitrate in acetonitrile solution, dried and activated, and developed with toluene at -20°C (Morris et al., 1967). Saturated FAME's were recovered and separated second time on C18-reversed phase TLC with acetonitrile/methanol/water 130:70:1 (v/v/v). Hydrogenation of unsaturated fatty acids were done by stirring samples in hydrogen gas

saturated air for 3 h in the presence of platinum(IV) oxide (PtO_2, Sigma) in methanol. Radioactivity distribution on the TLC plate was located and quantified with an Instant Imager (Packard Instrument Co., Meriden, CT).

Permanganate-periodate oxidation

In order to chemically cleave oleic acid at the double bond position permanganate-periodate oxidation (also called von Rudloff oxidative cleavage, Christie, 2003) was carried out. Oleic FAME band from aragentation-TLC was scraped into test tubes and eluted with 6mL of hexane:ethyl ether (2:1, v/v) and dried. The samples were redissolved in 1mL of 3.9mM K_2CO_3 in *t*-butanol/water (3/2) solution. After a thorough vortex, 1 mL of second solution containing (3.3mM $KMnO_4$, 102mM $NaIO_4$) was added and left for 1 h in room temperature with occasional vortex. Samples were acidified by few drops of 5M aqueous H_2SO_4 and then sulphurous acid (H_2SO_3) solution was added until yellow-green color persisted. The aqueous phase was extracted 2 times with 3mL diethyl ether and water was removed through anhydrous Na_2SO_4. After carefully evaporating to dryness the resulting nonanoic acid (C9A) and 1,ω-nonane-dioic monomethyl ester fragments (C9AE) were separated by K6 silica TLC using hexane: diethyl ether: acetic acid (90:10:1, v/v/v) system. Percent total radioactivity in each lane was quantified using the Instant Imager.

Acyl-ACP intermediate analysis

Modified version of procedure described by Mancha et al. (1975) was used to isolate acyl-ACP. Frozen samples were rapidly quenched on a heating block (> 90°C) with addition of hot isopropanol (equal volume with reaction) for 10 minutes. Lipids were extracted 2 times with 3 mL hexane. Precipitation of acyl-ACP fraction was carried out by addition of 50μL saturated ammonium sulfate solution and 8 mL of chloroform / methanol (1/2, v/v) at room temperature for 20 minutes. Pellet was collected by centrifugation (2,600 g for 5 minutes) and was washed again with 5 mL chloroform / methanol (1/2, v/v), 1% dimethoxypropane. The combined supernatant (13mL) was partitioned against 7 mL of 0.2 M H_3PO_4 in 2M KCl and the chloroform layer was recovered. After evaporation under N_2 gas, acyl lipids were transmethylated with BF_3 in methanol and FAME were extracted twice with 3mL hexane and was combined with lipid extracts collected at the initial hexane extractions. Radioactivity of an aliquot was counted by liquid scintillation counter (Beckman Coulter Inc.) to determine acetate incorporation into the total lipids.

The pellet fraction was resuspended in 2mL of 0.5M $NaOCH_3$ (in methanol) and transmethylation was allowed to proceed at room temperature for 20 minutes. Two mL's of water and lipid carriers were added and FAME was extracted three times with 2.5mL hexane. An aliquot was counted with liquid scintillation counter. FAME was separated from unesterified FA's by K6 TLC (hexane/diethyl ether/acetic acid=90/10/1). Percent total radioactivity in each lane was quantified using the Instant Imager.

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CHAPTER 4

THE PREDICTED CANDIDATES OF *ARABIDOPSIS* PLASTID INNER ENVELOPE MEMBRANE PROTEINS AND THEIR EXPRESSION PROFILES: Search for lipid transporters of *Arabidopsis* plastid envelope

ABSTRACT

Plastid envelope proteins from the *Arabidopsis thaliana* nuclear genome were predicted using computational methods. Selection criteria were: first, to find proteins with NH_2-terminal plastid targeting peptides from all annotated open reading frames from *Arabidopsis thaliana*, second, to search for proteins with membrane spanning domains among the predicted plastidial targeted proteins, and third, to subtract known thylakoid membrane proteins. 541 proteins were selected as potential candidates of the *Arabidopsis thaliana* plastid inner envelope membrane proteins (*At*PEM candidates). Only 34% (183) of the *At*PEM candidates could be assigned to putative functions based on sequence similarity to proteins of known function (compared to the 69% function assignment of the total predicted proteins in the genome). Of the 183 candidates with assigned functions, 40% were classified in the category of 'transport facilitation' indicating that this collection is highly enriched in membrane transporters. Information on the predicted proteins, tissue-expression data from expressed sequence tags (EST's) and microarrays, and publicly available T-DNA insertion lines were collected. The data set complements proteomic based efforts in the increased detection of integral membrane proteins, low abundance proteins, or those not expressed in tissues selected for proteomic analysis. Digital northern analysis of EST's suggested that the transcript levels of most *At*PEM candidates were relatively constant among different tissues in contrast to stroma and the

thylakoid proteins. However, both digital northern and microarray analysis identified a number of *At*PEM candidates with tissue-specific expression patterns.

Fatty acid (FA) metabolism in a plant cell requires the coordination and the exchange of FA and FA-derived lipids between the stroma and the extraplastidial organelles across the double membrane of the plastid envelope. It is likely that specific membrane proteins are involved in these processes. Of the more than 120 members of the ATP Binding Cassette (ABC) transporter family reported in the Arabidopsis genome, 20 possess plastid targeting sequences and transmembrane domains. These were selected as candidates for plastid envelope lipid transporters. T-DNA insertion mutants for 11 selected candidates were obtained and analyzed for FA compositions in leaves and seeds and for lipid contents of seeds. There were no major changes in fatty acid composition in any of these mutants. However, insertions in two genes (At1g70610, At5g64940) consistently showed reduced oil contents (about 60-70% of wild type) in seeds.

INTRODUCTION

The plastid envelope double-membrane separates lipid metabolism within the plastid from extraplastidial lipid metabolism. There is a considerable flux of lipid exchange and intermixing between the plastid and the endoplasmic reticulum (Ohlrogge and Browse, 1995) and this flux involves lipid transport across the plastid envelope membranes. In this chapter bioinformatics and reverse genetics approaches are described that were taken in efforts to search for plastid envelope lipid transporters. This search consisted of two major parts. First, a genome wide analysis was made to create an inventory of Arabidopsis plastid inner membrane proteins and their expression patterns. Second, candidates for possible lipid transporters were selected and a preliminary characterization of T-DNA insertion mutants of those candidates was conducted.

Plastids exist in a wide range of differential forms, including proplastids, chloroplasts, etioplasts, amyloplasts, leucoplasts, and chromoplasts, depending on the developmental stage and function of the plant cells in which they reside. To a large extent, the types of plastids that are carried by cells determine the metabolic function and products of the particular plant tissue (Kirk and Tilney-Bassett, 1978).

One constant feature among the various types of plastids is the double membrane envelope structure that surrounds the organelle. The envelope, especially the inner envelope, effectively separates plastid metabolism from that of the cytosol. At the same time, almost all carbon and a major flux of other metabolites, various polypeptides, and

signals must move through the envelope to coordinate and integrate metabolism with the entire cell. Plastid envelopes contain protein transport machinery (Schnell, 1995; Cline and Henry, 1996; Heins et al., 1998), and are a major site for membrane biogenesis. Metabolite transporters from chloroplast and/or nongreen plastids accommodate the requirements of the different photosynthetic or heterotrophic tissues (Kammerer et al., 1998; Neuhaus and Emes, 2000). Membrane constituents (glycerolipids, pigments, prenylquinones) are synthesized on the envelope membrane as well as the porphyrin ring and phytyl chain used in chlorophyll synthesis, and the enzymes required for chlorophyll breakdown in senescing plastids (for review see Joyard et al., 1998). It is also hypothesized that fatty acids that are synthesized within the stroma are exported through a channeled system on the envelope (Pollard and Ohlrogge, 1999; Koo et al., 2004). The acyl group modification of lipids such as desaturation takes place on the inner envelope and lipid-derived signals are produced on the envelope membranes (Miquel and Browse, 1992).

Despite the importance and complexity of plastid envelope, only a small fraction of envelope proteins have been purified or characterized (Joyard et al., 1998). Recently, several groups have initiated proteomic studies to identify the constituents of plant subcellular organelles and membranes (Santoni et al.,1998; Sazuka et al., 1999; Seigneurin-Berny et al., 1999; Ferro et al., 2000; Peltier et al., 2000; Schubert et al., 2002). Most of these studies have been based on 1-dimensional or 2-dimensional electrophoretic methods followed by mass spectrometric analysis of peptides derived from fractionated proteins. The continued development and improvement of these

methods resulted in the identification of hundreds of proteins, particularly from organisms with sequenced genomes. Nevertheless, there are some limitations in current proteomic technologies. In particular, many membrane proteins are found in very low abundance and/or expressed only in certain tissues, making comprehensive samples difficult to obtain. In addition, many integral membrane proteins remain difficult to solubilize and often do not resolve well by gel electrophoresis or isoelectric focusing (Adessi et al., 1997; Santoni et al., 2000). Finally, trypsin, most frequently used to digest proteins prior to mass spectrometric analysis has relatively few sites in the more hydrophobic integral membrane proteins. Together, these limitations make it likely that a significant fraction of the integral proteins present in membranes will remain difficult to detect by most proteomic technologies.

One of the first steps in understanding the function of a gene is to determine the subcellular localization of the gene product (Somerville and Dangl, 2000). To augment the proteomic approaches described above, an additional opportunity for the discovery of putative localization of proteins is now possible due to complete sequencing of the *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative, 2000). To determine subcellular localization of plastidial proteins one can make use of certain features of plastid-targeted proteins. The plastid genome itself encodes only about 120 single copy genes and therefore must rely on proteins from the nucleus. The protein constituents that are encoded in the nuclear genome are synthesized in the cytoplasm as higher molecular weight precursors containing an amino-terminal transit peptide which is cleaved following entry into the plastid (Cline and Henry, 1996). These pre-sequences of nuclear-

encoded plastid proteins, even though not strictly conserved, share some common features that can be used to predict localization using computer algorithms (Emanuelsson et al., 1999). This in combination with transmembrane α-helical domain characteristics, namely charge bias and hydrophobicity (Sonnhammer et al., 1998; Krogh et al., 2001), make it possible to identify candidates for plastid membrane proteins. Considering the nature of predictors and the selection criteria used (discussed in the Result and Discussion) the collection described in this paper is expected to contain mostly inner envelope integral membrane proteins.

Evidence is emerging that some lipid transporters belong to the ABC transporter class of membrane proteins. For example, the peroxisomal long-chain fatty acid transporters in yeast (Pat1p/ Pat2p, Hettema and Tabak, 2000), animal (ALDp, Mosser et al., 1993, 1994) and plant (PXA1, PED3, CTS) (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002) are ABC transporters. The phosphatidylcholine translocators (mouse Mdr2, human MDR3), the N-retinlidine PE flippase (ABCR) and the cholesterol transporter (ABC1) in mammals are P-glycoproteins belong to the ABC transporter family (Ruetz and Gros, 1994; Allikmets et al., 1997; Borst et al., 2000). The *E.coli* 'lipid A' transporter in the inner membrane (msbA) also belongs to the multidrug resistant protein subfamily of ABC transporter superfamily (Zhou et al., 1998). The Arabidopsis TGD1, involved in the lipid transfer from ER to plastid is similar to an ABC domain-lacking permease half-molecules of multipartite bacterial ABC transporter complexes (Xu et al., 2003). The Arabidopsis ABC transporter family consists of more than 120 members (Sanchez-Fernandez et al., 2001). In this study I present preliminary characterization of

T-DNA knockouts of several ABC transporters that are predicted as plastid inner membrane proteins.

RESULTS AND DISCUSSION

Part I. The Predicted Candidates of *Arabidopsis thaliana* Plastid Inner Envelope Membrane Proteins and Their Expression Profiles

Plastid Envelope Membrane Protein Candidates

The strategy to select candidates of plastid envelope integral membrane proteins from Arabidopsis nuclear genome sequences by computational methods and the results are summarized in Figure 20.

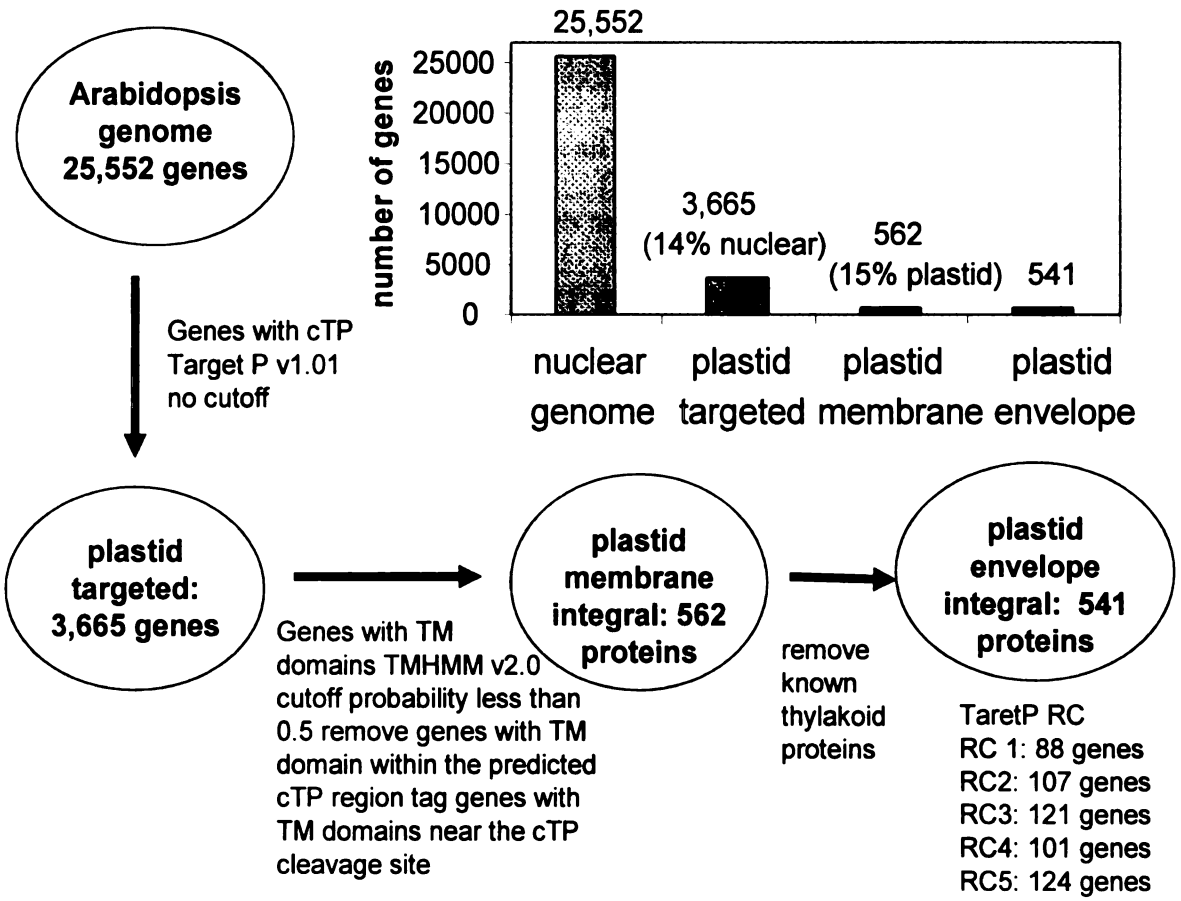
The first step was to predict plastid targeted proteins from the entire Arabidopsis genomic sequences (See Methods and Materials for Arabidopsis genomic sequence retrieval). This was done using TargetP (http://www.cbs.dtu.dk/services/TargetP/). TargetP is considered the best subcellular location predictor that is publicly available (Emanuelsson and von Heijne, 2001; Bannai et al., 2002; Peltier et al., 2002) and was also chosen by the Arabidopsis Genome Initiative to analyze the recently finished genome sequence of *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000). Protein targeting to the plastid usually relies on its amino terminal pre-sequence (Cline and Henry, 1996) and TargetP, a neural network-based tool, is trained to recognize those signals. Overall

success rate on test sets analyzed by the developers was 85% (Emanuelsson et al., 2000). The sensitivity for chloroplast-targeted proteins was 85% while the specificity was lower (69% and 84% on two different test sets). This means that more false positives are expected than the false negatives. In order to increase the specificity, cut off restraints can be applied. However, in order to include a maximum number of possible candidates we instead chose default decision rule (winner takes all) and designated in our database the 'reliability classes' from 1 to 5 (Emanuelsson et al., 2000). In total 3,665 out of 25,552 (14.3%) proteins encoded by the Arabidopsis nuclear genome were predicted to be plastidial which is similar to the reported value of 3,574 from the Arabidopsis Genome Initiative (The Arabidopsis Genome Initiative, 2000).

These 3,665 predicted plastid targeted proteins were next analyzed for transmembrane α-helical domains (Figure 20). There are several web-based predictors available (Moller et al., 2001). A recent study compared the performance of 14 different methods, against 883 membrane spanning regions of biochemically characterized proteins, and concluded that TMHMM is the most accurate method for transmembrane segment detection (Moller et al., 2001). Subsequently, HMMTOP also has been shown to have similar performances (Moller et al., 2002). Most recent methods for prediction of transmembrane helices rely on hydrophobicity and charge bias of the transmembrane regions. There were variations in the accuracy of overall correct topology prediction (number and location and orientation of transmembrane regions), however correct prediction of the presence of

Figure 20. Summary scheme of plastid envelope protein prediction from *Arabidopsis thaliana* nuclear genome.

The graph displays the number of proteins following each selection steps. Subcellular localization was predicted using TargetP v1.01 web server. Transmembrane region prediction was done by TMHMM v2.0 web server. cTP: chloroplast transit peptide. RC: TargetP reliability class. TM: transmembrane domain. See Materials and Methods for web site addresses for the predictors.



transmembrane segments *per se* was overall 95% percent in a comparison of 3 best predictors (Sonnhammer et al., 1998). TMHMM is reported to discriminate between soluble and membrane proteins with both specificity and sensitivity better than 99% (Krogh et al., 2001). TMHMM, like Target P is also a neural network based program but built with an architecture that corresponds more closely to the biological system (Hidden Markov Model). The probability of a predicted integral protein to be a true membrane protein is positively correlated with the expected number of amino acid residues within the transmembrane segments and also with the expected number of transmembrane segments in the protein. In other words the more amino acid residues per predicted transmembrane segments and the more predicted number of transmembrane segments per gene protein, the more likely that it is a truly integral protein. In order to include as many integral proteins as possible we selected all proteins that have at least one predicted transmembrane domain regardless of amino acid residue numbers. One of the advantages of TMHMM, is that it provides plots of probabilities for prediction throughout the analyzed proteins. In our study, proteins that were overall not predicted to be transmembrane proteins yet contained weak α-helical domains with probabilities over 50% were also included in the database as potential membrane proteins with their probabilities. Because the accuracy of TMHMM prediction drops when signal peptides are present (Krogh et al., 2001), transmembrane predictions within the predicted targeting peptides (according to the TargetP cleavage site predictions) were removed and those domains which were predicted close to the targeting peptides were designated in the database as 'N-term'. After processing as described above, out of 3,665 predicted

plastidial proteins 562 (about 15%) contained potential transmembrane α-helical domains (Figure 20) and thus are considered as plastid membrane protein candidates.

The third step was to eliminate the thylakoid integral membrane proteins. Although there are suborganelle location predictors such as PSORT the overall accuracy of such programs is not high enough (about 50%) to use for discrimination between the thylakoid and the envelope localized proteins (Nakai et al., 1999). It is estimated that there are at least 200 proteins in the lumenal space and in the periphery of the thylakoid membrane (Peltier et al., 2000). Four multisubunit protein complexes with 75 to 100 peptides involved in photosynthesis, comprise the majority of the thylakoid membrane proteome (Peltier et al., 2000). These proteins are relatively well studied and many of them are known to be encoded by the plastid genome which contains about 90 protein encoding genes (Sugita and Sugiura, 1996). If the known nuclear encoded thylakoid membrane proteins are removed from our candidate list of 562, the remaining candidates are expected to be highly enriched in envelope proteins. After manual removal of 21 known thylakoid associated proteins and their homologs, 541 proteins remained as potential candidates for *Arabidopsis thaliana* Plastid Envelope Membrane proteins (*At*PEM).

It is important to note that these are predicted to be integral membrane protein candidates and the candidate list will contain omissions and inclusions of various types. First, as mentioned above, the specificity of Target P prediction is lower than the sensitivity. Because we did not employ any specificity cut off for Target P prediction and used low stringency for transmembrane prediction (transmembrane domain probability > 50%),

these 541 candidates may contain many false positives. If instead, the Target P specificity is set to > 0.95 and proteins with low probabilities of transmembrane domain are excluded, about 250 candidates remain and these represent a higher reliability core set. These higher reliability candidates are color coded on our website. Secondly, there will be many membrane proteins that do not have transmembrane domains but are associated with the envelope through different types of interactions such as β-sheet conformation, polyisoprenylation, acylation, glycolipid anchors, protein-protein interaction mediated associations, etc. Thirdly, most outer membrane localized proteins appear to insert directly into the outer envelope membrane without going through the general import apparatus (Cline and Henry, 1996; Fulgosi and Soll, 2001; Schleiff and Klosgen, 2001). Therefore, the 541 *At*PEM candidates will under represent the outer membrane proteins. However, the protein composition of outer membranes is much simpler than that of inner membrane. The lipid to protein ratio of outer membrane is about 3 times higher than that of inner membrane and the inner envelope membrane, which contains various metabolite translocators, is the main permeability barrier for solutes although there is growing evidence suggesting that the outer envelope may contain regulated ion channels (Cline et al., 1981; Block et al., 1983; Douce and Joyard, 1990; Pohlmeyer et al., 1998; Flugge, 2000; Bolter and Soll, 2001). Thus, we consider it likely that *At*PEM candidates represent the majority of integral plastid envelope proteins.

What are they?

Figure 21A indicates the functional identification status for the selected 541 *At*PEM candidates based on the short descriptions in the annotation databases (MIPS and GenBank). The proteins with identified function (identified) or that have homology with known proteins (X-like, putative-X) comprised only about 29% of the 541 whereas 71% of the candidate proteins could not be assigned to any known function (putative, unknown, hypothetical). This is a very low characterized status compared to the overall 69% functional assignment of the whole genome (The Arabidopsis Genome Initiative, 2000) and reflects the past difficulties in studying membrane proteins (Wilkins et al., 1998; Seigneurin-Berny et al., 1999).

More detailed functional classification of the *At*PEM candidates was done according to the automatically derived functional categories from MIPS *Arabidopsis thaliana* Data Base using Protein Extraction Description and Analysis Tool (PEDANT) web server (http://pedant.gsf.de/, Frishman et al., 2001). Among the 541 candidates, 183 (34%) were found in the PEDANT database and were classified into 17 classes and 89 subclasses. Figure 21B shows the number of *At*PEM candidates found in each category. The largest number of *At*PEM candidates (39% of 183 classified predicted proteins) fell into the class of 'transport facilitation'. Thus membrane transporters are highly enriched in the selected *At*PEM candidates. Figure 21C shows the sub-classification of 'transport facilitation' and 'metabolism' classes. The 3 major subclasses for 'transport facilitation' were 'ion transporters', 'C-compound and carbohydrate transporters' and 'ABC (ATP binding cassette) transporters'.

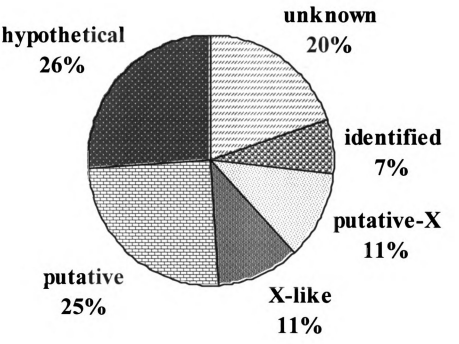
There were also several candidates involved in protein translocation into the plastids. None of the outer membrane components of the protein translocon were found among *At*PEM candidates as was expected (many outer membrane proteins lack obvious plastid targeting sequences as discussed above) whereas 5 Arabidopsis homologs to the known pea translocon at the inner membrane of chloroplast (Tic)110, Tic20, Tic40, Tic55 (Jackson-Constan and Keegstra, 2001) were present among the candidates (Table 7). Two Arabidopsis homologs of Tic22 were predicted to be targeted to the plastids by the TargetP but did not have obvious transmembrane spanning domain (by TMHMM prediction). Tic22 is thought to be peripherally associated with the inner envelope membrane facing the intermembrane space (Jackson-Constan and Keegstra, 2001).

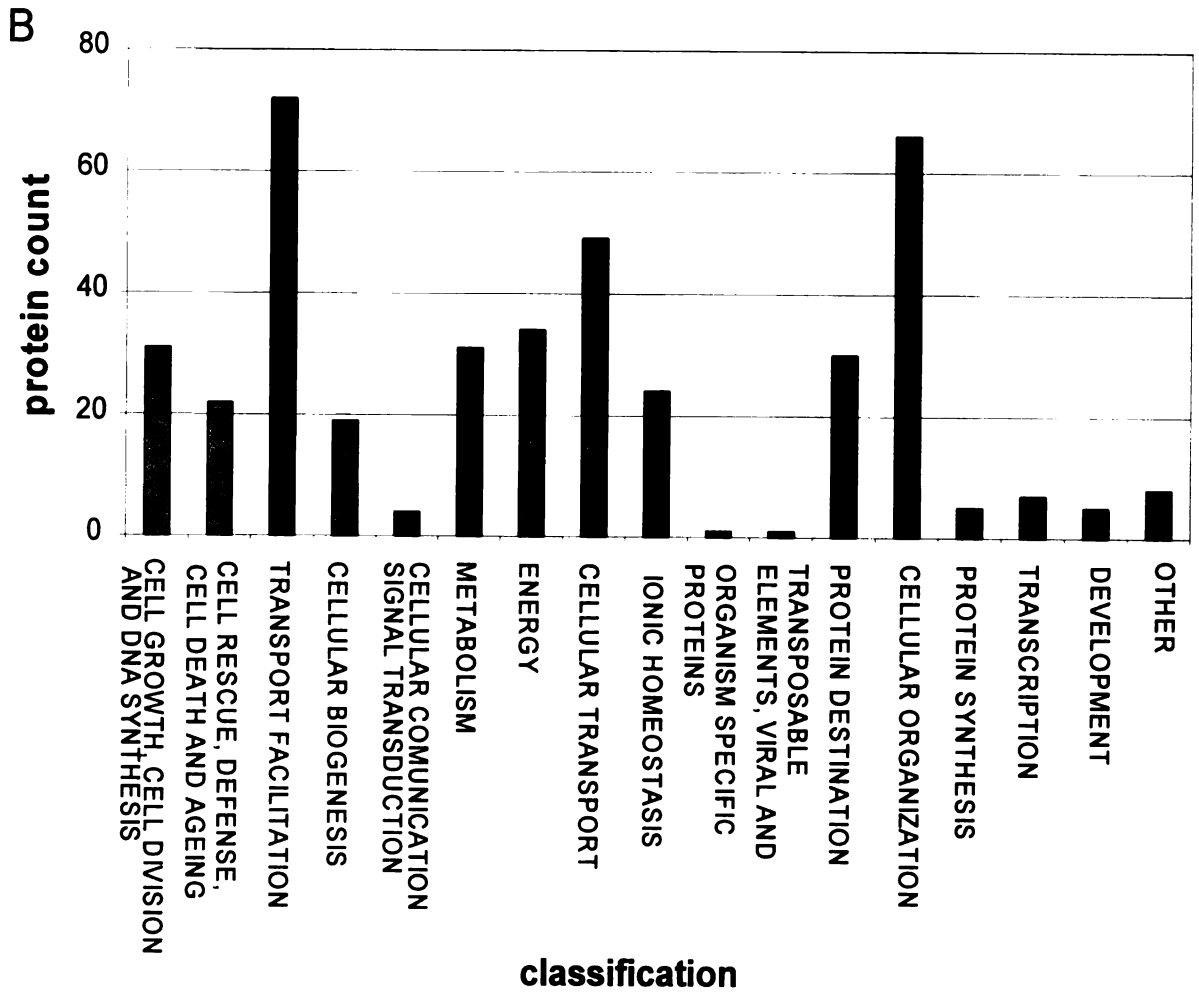
In agreement with the known role of plastid envelopes in lipid metabolism, 'lipid, fatty-acid and isoprenoid metabolism' related proteins represented the largest sub-set among the 'metabolism' class. Table 7 presents the result of a more detailed survey of proteins predicted or known to be involved in plant lipid metabolism (search was carried out using our database of predicted lipid related genes in *Arabidopsis thaliana* (Mekhedov et al., 2001; Beisson et al., 2003)). Seven proteins potentially responsible for known enzymetic reactions to produce glycerol lipids in the plastid envelope were found among *At*PEM candidates (Table 7, for review see Ohlrogge and Browse, 1995). Arabidopsis homologs of plastidial 2-lysophosphatidic acid acyltransferase (EC 2.3.1.51) which mediates acyl group transfer from acyl-ACP (acyl carrier protein) to sn-2 position of lyso-phosphatidic acid (1-acyl-sn-glycerol 3-phosphate) to produce phosphatidic acid

Figure 21. Functional classification of the Arabidopsis plastid envelope protein (*At*PEM) candidates.

(A) Overall functional annotation status based on the short descriptions of each candidates from MIPS (chromosome 1,3,5) and from GenBank (chromosome 2,4). X: any protein/gene/enzyme name. (B) Detailed functional classification using automatically derived functional categories from PEDANT web server (see Materials and Methods). (C) Subclassification of the proteins classified as 'transport facilitation' and 'metabolism' by PEDANT.

A




B
protein count
80
60
40
20
0
CELL GROWTH, CELL DIVISION AND DNA SYNTHESIS
CELL RESCUE, DEFENSE, CELL DEATH AND AGEING
TRANSPORT FACILITATION
CELLULAR BIOGENESIS
CELLULAR COMUNICATION SIGNAL TRANSDUCTION
METABOLISM
ENERGY
CELLULAR TRANSPORT
IONIC HOMEOSTASIS
ORGANISM SPECIFIC PROTEINS
TRANSPOSABLE ELEMENTS, VIRAL AND
PROTEIN DESTINATION
CELLULAR ORGANIZATION
PROTEIN SYNTHESIS
TRANSCRIPTION
DEVELOPMENT
OTHER
classification

C.

Class	Subclass	no. of genes
TRANSPORT FACILITATION		
	ion transporters	24
	C-compound and carbohydrate transporters	22
	ABC transporters	16
	other transporter facilitators	15
	Channels/pores	9
	amino-acid transporters	8
	drug transporters	6
	purine and pyrimidine transporters	6
	Transport ATPases	2
	peptide-transporters	1
METABOLISM		
	lipid, fatty-acid and isoprenoid metabolism	8
	nucleotide metabolism	6
	C-compound and carbohydrate metabolism	5
	metabolism of vitamins, cofactors, and prosthetic groups	5
	nitrogen and sulfur metabolism	5
	phosphate metabolism	4
	amino acid metabolism	3
	secondary metabolism	3

Table 7. *At*PEM candidates involved in protein import and glycerolipid metabolism.

Protein / Enzyme Name	locus ID	Description
plastid envelope protein import machinery [a]		
Tic 110	At1g06950	Chloroplast inner envelope protein, putative
Tic 55	At2g24820	putative Rieske iron-sulfur protein
Tic 20	At1g04940	unknown protein
	At4g03320	putative chloroplast protein import component
Tic 40	At5g16620	translocon Tic40-like protein
glycerolipid biosynthetic enzymes [b]		
Plastidial cytidine-5'-diphosphate diacylglycerol synthase	At4g26770	putative CDP-diacylglycerol synthetase
	At3g60620	phosphatidate cytidylyltransferase - like protein
Plastidial hosphatidylglycerophosphate synthase	At2g39290	putative CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
Monogalactosyldiacylglycerol synthase	At4g31780	monogalactosyldiacylglycerol synthase-like protein
Plastidial oleate desaturase (FAD6)	At4g30950	chloroplast omega-6 fatty acid desaturase (fad6)
Plastidial linoleate desaturase (FAD7/FAD8)	At5g05580	temperature-sensitive omega-3 fatty acid desaturase, chloroplast precursor
	At3g11170	omega-3 fatty acid desaturase, chloroplast precursor
ABC transporters [c]		
Full Molecule ABC Transporters	At1g15210	putative ABC transporter
	At1g15520	ABC transporter, putative
	At1g59870	ABC transporter, putative
	At1g66950	ABC transporter, putative
	At3g16340	putative ABC transporter
	At3g21250	unknown protein
Half-Molecule ABC Transporters	At1g70610	putative ABC transporter
	At1g54350	ABC transporter family protein
	At2g39190	putative ABC transporter
	At2g28070	putative ABC transporter
	At3g25620	membrane transporter, putative
	At3g52310	ABC transporter, putative

Table 7. continued

Protein / Enzyme Name	locus ID	Description
	At5g03910	ABC transporter -like protein
	At5g19410	membrane transporter - like protein
	At5g52860	ABC transporter-like protein
	At5g64940	ABC transporter-like

[a] Arabidopsis homologs of the pea plastid protein import machinery were according to the published data by Jackson-Constan and Keegstra (2001).

[b] Arabidopsis glycerolipid biosynthetic enzyme candidates were searched using our database of lipid metabolism related genes in *Arabidopsis thaliana* (Mekhedov et al., 2001; Beisson et al., 2003).

[c] Search for the ABC transporters was done using the complete inventory of ABC protein superfamily in Arabidopsis from the website at http://www.arabidopsisabc.net/, Sanchez-Fernandez et al., 2001).

(1,2-diacyl-sn-glycerol 3-phosphate) was missing. All three Arabidopsis homologs of this enzyme were predicted to a different subcellular location ('mitochondria' and 'others') by TargetP. Digalactosyldiacylglycerol synthase (EC 2.4.1.184) which was known to localize on the outer envelope (Froehlich et al., 2001) was not among the *At*PEM candidates. Although it had plastid transit peptide recognized by TargetP, no membrane spanning region was detected by TMHMM.

Surprisingly there were several candidates that are similar to proteins involved in cell wall biogenesis and modifications, such as arabinogalactan-protein homolog, cellulose synthase catalytic subunit -like protein, pectinesterase - like protein, pectin

methylesterase-like protein, putative xyloglucan fucosyltransferase, etc. These unexpected relationships between the plastid envelope and predicted proteins annotated with functions that are generally considered non-plastidial may reflect inaccurate prediction by TargetP software. Alternatively, because these putative functions are assigned based solely upon sequence similarities, an equally likely possibility is that the annotations point toward related, but previously un-described functions in the plastid. This incongruence therefore represents an example where bioinformatics analysis can direct attention toward potential novel functions in the plastid envelope.

Characteristic Features of Plastid Envelope Candidates

The distribution of the peptide length of *At*PEM candidates is shown in Figure 22A. 80% of the candidates were smaller than 600 amino acids in length. The average length of the peptides was 411 amino acids which is slightly smaller than the average peptide length of 434 amino acids for the nuclear genome (The Arabidopsis Genome Initiative, 2000). As indicated in Figure 22B, 63% of the *At*PEM candidates contained 2 or less membrane spanning domains whereas there were 40 candidates that had 10 or more membrane spanning domains. The membrane spanning domains of proteins with only a few such domains might serve as an anchor to the envelope or to tether the peripheral proteins. Interestingly 70% of candidates with 10 or more membrane spanning domains were classified as 'transport facilitation'. Due to their high hydrophobicity these potential transporters would be particularly difficult to display on 2 dimensional electrophoretic gels of purified plastid envelopes.

*At*PEM candidates provide an opportunity to compare predicted plastid envelope proteome with the proteome of other organisms, i.e., Synechocystis. The Synechocystis genome contains about 3,167 protein encoding genes which is roughly the number predicted to be plastidial in Arabidopsis. Highly similar proteins for about 32% (175) of *At*PEM candidates were in the Synechocystis genome (data not shown) compared to 44% and 47% of thylakoid and stromal proteins, respectively. Thus, the boundary envelop of the plastid may have a more "mixed" evolutionary origin than the internal components.

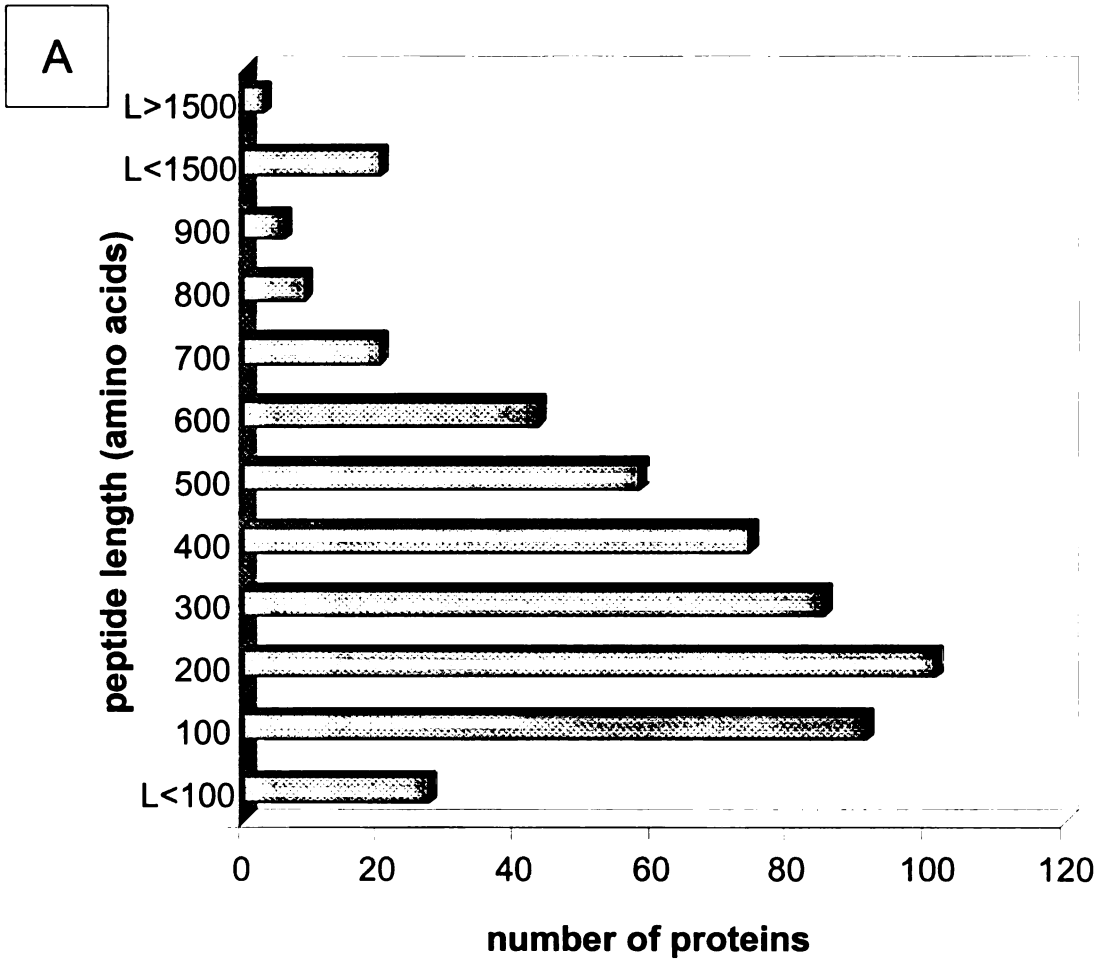
We also compared our list of candidates to proteins identified by mass spectrometric analysis of Arabidopsis chloroplast envelope preparations (Froehlich et al., 2003; Ferro et al., 2002, 2003). In Table 8 are listed 37 *At*PEM candidates that are represented in the proteins identified through proteomics approach by both groups. The proteomics approach identified many non-integral membrane proteins and outer membrane proteins not selected by our approach. Thus, the bioinformatic identifications reported in this study clearly provide novel information on a set of inner envelope integral membrane proteins that are not easily characterized by proteomics.

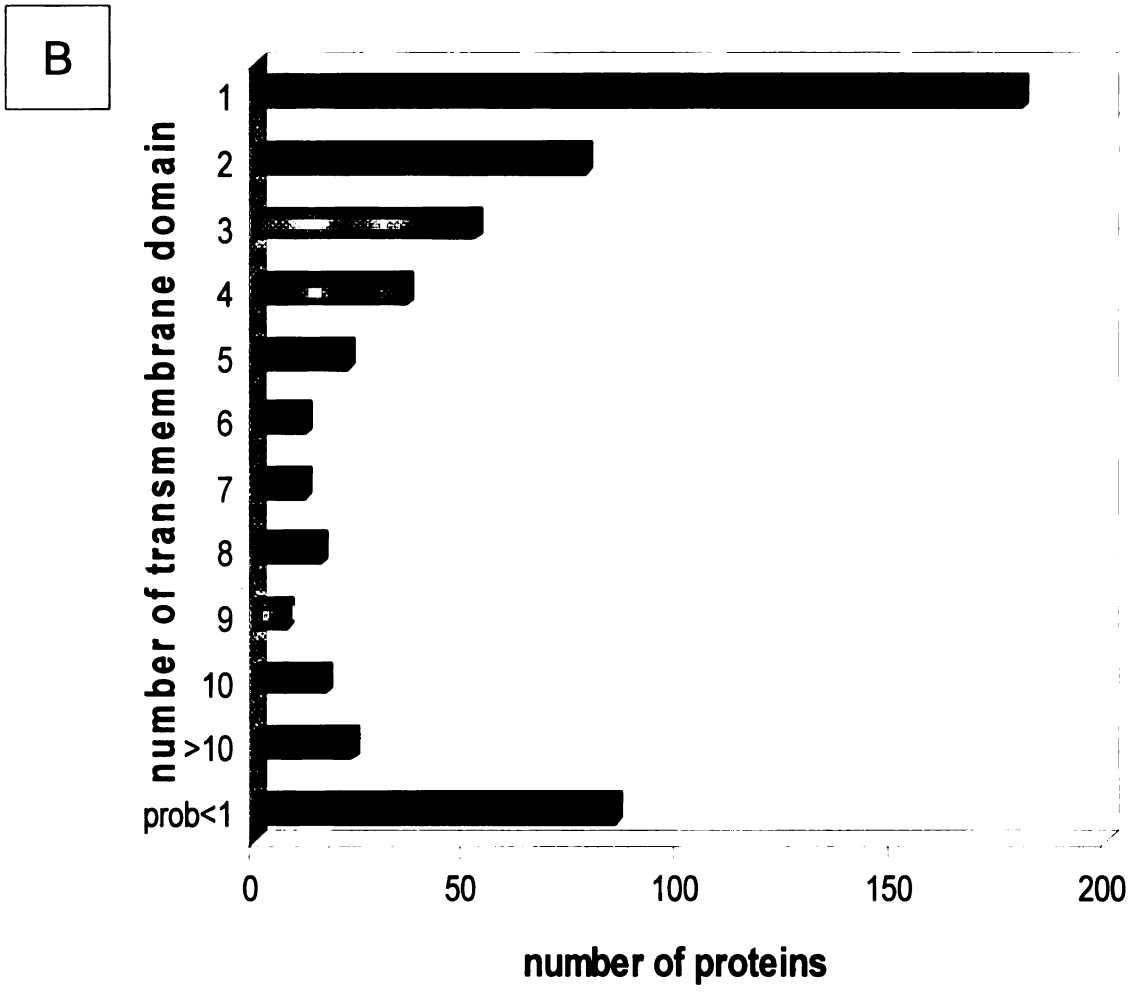
Digital mRNA Expression Profile

In addition to the subcellular localization, indirect information on cellular or developmental function can be obtained from spatial and temporal expression patterns of genes. Gene chips, microarrays, Expressed Sequence Tags (ESTs), and Serial Analysis of

Figure 22. Characteristics of *At*PEM candidates.

(A) Peptide length distribution. L: number of amino acid residues. Plastid targeting sequences are removed based on TargetP cleavage site predictions. (B) Distribution of the number of membrane spanning domains. TMHMM v2.0 web server was used for the membrane spanning domain prediction. prob<1, proteins with spanning domain probability less than 1.




B
number of transmembrane domain
1
2
3
4
5
6
7
8
9
10
>10
prob<1
0
50
100
150
200
number of proteins

Gene Expression (SAGE) all provide useful means to study mRNA expression profiles (Bouchez and Hofte, 1998).

A large proportion (68%) of *At*PEM candidates have at least 1 EST in the GenBank dbEST (113,330 ESTs as of January 4, 2002, Table 9) and 87% (470/541) were represented by The Institution of Genomic Research (TIGR) Tentative Consensus (TC) sequences (163,752 TCs as of May 23, 2001, http://www.tigr.org/tdb/agi/ ,Quackenbush et al., 2000, see Materials and Methods. Thus the majority of the *At*PEM candidates are transcriptionally active.

The abundance of ESTs sequenced from different cDNA libraries can provide an estimate of relative transcript abundance provided a number of conditions are met (Audic and Claverie, 1997). To obtain tissue-specific expression profiles of *At*PEM candidates, 110,000 ESTs deposited in GenBank and sequenced from 55 different cDNA libraries were grouped into 8 'library pools' according to the source tissues from which the cDNA libraries were derived (Table 9, Beisson et al., 2003). We then surveyed the abundance of ESTs within each library pool for *At*PEM candidates after normalization by dividing the number of *At*PEM candidate ESTs in the given 'library pools' by the total number of ESTs of that 'library pool'. The relative abundances of ESTs for *At*PEM candidates in each tissue were similar, ranging from 1.4 to 2.5 % of the total ESTs, except for the 'flowers' pool which showed the highest abundance at 4.2% of ESTs. Although these aggregate EST frequencies varied little, a number of individual proteins were found to have more distinct tissue-specific expression patterns. In order to study this in detail,

Table 8. The *At*PEM candidates represented in the chloroplast envelope proteins identified through protein mass spectrometry by Ferro et al. (2002, 2003) and by Froehlich et al. (2003).

locus ID	amino acid	RC[a]	TM[b]	probability[c]	ESTs	Annotation
At1g06950	189	1	2	1	5	Tic110 homologue
At1g20830	349	2	1	1	1	hypothetical protein
At1g32080	512	1	12	1	2	unknown protein
At1g42960	168	2	1	1	8	hypothetical protein
At1g67080	220	1	4	1	2	hypothetical protein
At1g78620	333	2	3	1	9	hypothetical protein
At2g24820	539	1	2	0.8		AC006585_25 putative Rieske iron-sulfur protein
At2g34460	280	4		0.5	6	unknown protein
At2g38550	327	4	3	1	3	putative non-green plastid inner envelope membrane protein
At2g39290	296	4	1	1	3	putative CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
At2g47840	208	3	4	1	1	hypothetical protein
At3g11170	446	2	3	1	14	omega-3 fatty acid desaturase, chloroplast precursor
At3g20320	381	1	1	1	5	unknown protein
At3g47060	802	3		0.8	3	FtsH metalloprotease - like protein
At3g51870	381	5	2	1	4	putative carrier protein
At3g57280	239	2	3	1		hypothetical protein
At3g60590	329	3	5	1	4	putative protein
At3g61870	272	4	4	1	7	putative protein
At3g63410	338	1	1	1	44	putative chloroplast inner envelope protein

Table 8. continued.

locus ID	amino acid	RC[a]	TM[b]	probability[c]	ESTs	Annotation
At4g25650	548	1	3	1	8	Putative protein
At4g30950	448	2	4	1	11	chloroplast omega-6 fatty acid desaturase
At4g31780	533	2	1	1	4	monogalactosyldiacylglycerol synthase
At4g39460	330	4	3	1	11	mitochondrial carrier-like protein
At5g01500	415	3		0.7	4	putative protein
At5g12860	557	1	13	1	21	2-oxoglutarate/malate translocator precursor -like protein
At5g13720	262	2	3	1	3	unknown protein
At5g16150	560	5	10	1	2	sugar transporter-like protein
At5g16620	447	2		0.5	10	translocon Tic40-like protein
At5g17520	347	1	7	1	2	root cap 1 (RCP1)
At5g19750	288	2	2	1	2	putative protein
At5g22790	433	1	1	1	1	putative protein
At5g23890	946	3	1	1	5	unknown protein
At5g33320	408	1	6	1	38	phosphate/phosphoenolpyruvate translocator precursor
At5g52540	461	1	10	1	8	putative protein
At5g59250	579	1	9	1	3	D-xylose-H+ symporter - like protein
At5g62720	243	3	4	1	9	putative protein
At5g64290	563	2	9	1	7	2-oxoglutarate/malate translocator

[a] 'Reliability Class' of the subcellular localization prediction provided by the TargetP web server.

[b] number of spanning region predicted by TMHMM web server.

[c] probability of membrane spanning domain provided by TMHMM web server.

Table 9. Summary of ESTs of *At*PEM candidates by tissue types.

	Tissue Specific Library Pools [a]								
	Cell suspension	Flowers	Leaves	Mixed	Roots	Seedlings	Seeds	Siliques	SUM
Library size	986	7566	3774	42855	18350	6058	10678	12842	103109
no. of ESTs [b]	14	321	91	1061	319	153	158	232	2349
percent total ESTs [c]	1.4	4.2	2.4	2.5	1.7	2.5	1.5	1.8	2.3
no. of candidates with ESTs [d]	11 (2)	99 (18)	78 (14)	249 (46)	152 (28)	75 (14)	89 (16)	127 (24)	365 (68)

[a] EST libraries were grouped into 8 'library pools' according to the source tissues where the cDNA library was derived from (Beisson et al., 2003).

[b] Number of ESTs corresponding to the *At*PEM candidates found in each library pool.

[c] Percent total ESTs in the library.

[d] Number of *At*PEM candidates that has at least one EST in given library pool. Percent total number of *At*PEM candidates are within the parentheses.

tissue-specific expression patterns of individual candidates were analyzed using statistical equations developed by Audic and Claverie (http://igs-server.cnrs-mrs.fr) which differentiate between random EST sampling fluctuations versus significant change inEST frequencies. The number of ESTs corresponding to a given gene (*At*PEM candidate) found in each of three tissue-specific library pools ('flowers', 'roots', or 'seeds') was compared with that in the reference 'mixed' pool and the results are presented in Table 10. A total of 21 *At*PEM candidates (6% of 365 candidates that had at least 1 EST) displayed tissue-specific expressions when $p<0.005$ (p, a probability of the compared EST abundances being different by chance) was applied. Figure 23 visualizes the digital expression profile of 21 *At*PEM candidates that showed tissue-specific expression pattern at $p<0.005$. Among the differentially expressed genes were glucose-6-phosphate/phosphate translocator (GPT) and phosphoenol pyruvate/phosphate translocator (PPT). GPT was previously shown to be highly expressed in developing maize kernel and potato tuber (Kammerer et al., 1998) and the ESTs of GPT were abundant in Arabidopsis developing seed EST database (White et al., 2000). In agreement with these observations, GPT had higher EST abundance in 'seeds' library pool than the reference at $p<0.004$. Similarly, for PPT, biochemical analysis and mRNA blotting results indicated a high expression of PPT in non-green tissue (Kammerer et al., 1998) especially roots (Fischer et al., 1997) and in our EST analysis, PPT expression was highest in the 'roots' ($p<0.003$). Thus, the comparisons in Table 10 agree with previous biochemical characterizations and support the validity of the digital expression profile approach. Many of the proteins shown in Table 10 are of unknown function, including several with high representation in specific tissues.

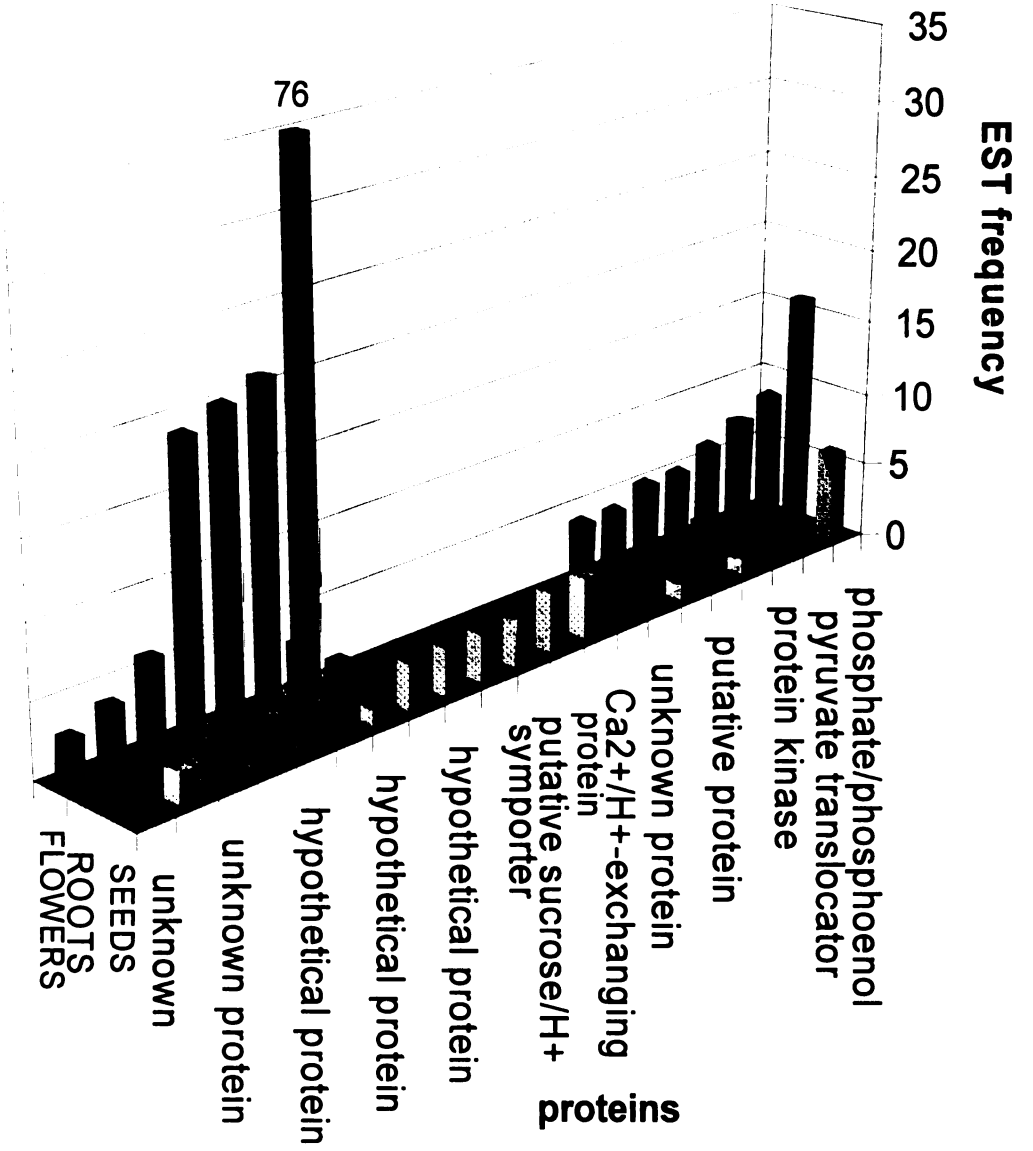
Table 10. Summary of *At*PEM candidates with tissue-specific transcript abundances.

Number of ESTs in the 'flowers', 'roots', 'seeds' pools was compared with that in the 'mixed' pool. Candidates with probabilities of EST frequency differences being by chance less than 0.005 are grouped according to the tissue specificity of the transcript abundances (shown in the left shaded column). [a] F: Flowers, R: Roots, S: Seeds, M: Mixed. Probability calculations were according to Audic and Claverie, 1997 (see Materials and Methods).

	Locus ID	Descriptions	Number of ESTs				Probabilities[a]		
			F	R	S	M	F/M	R/M	S/M
Flower	At1g08380	unknown protein	19	1	0	10	0.001	0.2	0.2
	At1g20340	hypothetical protein	21	5	0	41	0.001	0.004	0.001
	At1g30380	hypothetical protein	20	1	0	12	0.001	0.1	0.2
	At2g06520	hypothetical protein	76	3	1	36	0.001	0.002	0.005
	At2g15290	unknown protein	4	1	2	2	0.003	0.7	0.2
	At1g74730	unknown protein	3	1	0	1	0.005	0.5	0.8
	At2g26500	unknown protein	6	0	0	7	0.005	0.2	0.4
Root	At1g15210	putative ABC transporter	0	10	0	2	0.8	0.001	1
	At2g40380	unknown protein	0	6	0	0	0.4	0.001	0.4
	At3g24550	protein kinase, putative	1	9	0	2	0.3	0.001	1
	At4g01750	hypothetical protein	0	6	0	0	0.4	0.001	0.4
	At5g13390	putative protein	0	7	0	1	0.6	0.001	0.8
	At5g16910	cellulose synthase catalytic subunit - like protein	0	7	1	1	0.6	0.001	0.3
	At5g33320	phosphate/phosphoe nol-pyruvate translocator precursor	0	16	6	12	0.3	0.003	0.2
	At3g03050	putative cellulose synthase catalytic subunit	0	8	1	3	1	0.004	0.6
Seed	At3g51860	Ca2+/H+-exchanging protein-like	0	1	4	0	0.4	0.2	0.001
	At1g09960	putative sucrose/H+ symporter	0	0	3	0	0.4	0.6	0.004
	At1g61800	hypothetical protein	0	0	3	0	0.4	0.6	0.004
	At4g07990	hypothetical protein	0	0	3	0	0.4	0.6	0.004
	At5g52860	ABC transporter-like protein	0	0	3	0	0.4	0.6	0.004
	At5g54800	glucose-6-phosphate/phosphate translocator	0	0	4	1	0.6	1	0.004
		EST library size	7566	18350	10678	42855			

Figure 23. Digital differential display analysis.

*At*PEM candidates with statistically different levels ($p<0.005$, where p is probability of difference being by chance) of EST frequencies are displayed. Number of ESTs in the 'Flowers', 'Roots' or 'Seeds' pools was compared with that in the reference 'mixed' library pool. The bars indicate the abundances of the ESTs (EST frequency axis) corresponding to the proteins (proteins axis) in each library pools (tissue axis).



The bioinformatics predictions of suborganellar localization together with tissue-specific expression provides initial clues that may help in discovering the functions of these proteins. However, it should be noted in such analysis that the normalization procedures used in the construction of the cDNA libraries for EST projects can undermine the statistical analysis and even with the high probability such as $p<0.005$ used in Table 10, there could still be false positives (Audic and Claverie, 1997). Therefore, the digital expression profile analysis should be considered as an initial step to find possible candidates for differentially expressed genes from a large collection of data and needs to be verified by experiments such as northern blots, RT-PCR (Reverse Trascription – Polymerase Chain Reaction) or western blots, etc.

Plastids undergo massive changes during differentiation into their various forms (chloroplast, chromoplast, amyloplast, leucoplast, etioplast, etc.) and need to import different spectra of proteins according to their changed biochemical properties. However the structure of the concentric pair of envelope membranes remains constant (Douce and Joyard, 1990; Joyard et al., 1998). It is yet unknown the extent to which the proteome of the envelope will also remain unchanged. The low number (21) of candidates differentially expressed between the different tissues may indicate that the envelope proteome candidates are relatively constitutive in terms of transcript abundance across the different tissues. Furthermore, when the tissue-specific EST distributions of *At*PEM candidates and that of 39 thylakoid localized proteins and 1,802 stromal proteins were compared, *At*PEM candidates showed the least changes (Table 11). Whereas only 8% of the *At*PEM candidates were tissue-specific at $p<0.05$, 69% of the analyzed thylakoid localized proteins showed specific EST tissue distributions. The stromal proteins also

displayed a higher level (19% at $p<0.05$) of differential expression than the *At*PEM candidates. Thus based on EST frequencies from different cDNA libraries, the transcript abundance for the envelope membrane proteins appears relatively constant compared to that of the stromal or thylakoid proteins.

Microarray Analysis

In order to further investigate the tissue-specific gene expression pattern of *At*PEM candidates, we analyzed publicly available cDNA microarray data from the Stanford Microarray Database (SMD, http://genome-www.stanford.edu/microarray, Sherlock et al., 2001) and from the Arabidopsis developing seed array website at http://www.bpp.msu.edu/Seed/SeedArray.htm (Girke et al., 2000; Ruuska et al., 2002). Expression profiles of 230 *At*PEM candidates for about 110 different microarray experiments were found in SMD public domain (as of March 12, 2002). We specifically examined tissue comparison data which were publicly available for comparisons between flower, leaf, or root versus the whole plant for 147 *At*PEM candidates and for seed versus leaf or seed versus plantlets for 59 *At*PEM candidates. Figure 24 presents a summary of genes which displayed more than 2 fold changes in at least 2 different tissue comparisons and the pattern of which agreed in all combinations (i.e. At1g29390 expression is higher in leaf compared to that in flower and higher in leaf also when compared to that in root). Twenty two candidates showed higher levels of transcript abundance in leaf when

Table 11. Tissue-specific transcript abundance comparison between the plastid envelope, thylakoid and stroma localized proteins.

	envelope	thylakoid	stromal
number of predicted proteins analyzed [a]	541	39	1802
number of ESTs [b]	2349	1961	17970
genes with altered EST tissue distribution [c]			
at probability < 0.05	8% (42)	69% (27)	19% (342)
at probability < 0.01	2% (13)	51% (20)	4% (71)

[a] Predicted envelope localized proteins were from *At*PEM candidates. Thylakoid proteins were selected from known thylakoid proteins and their homologs mostly involved in photosynthesis. Stromal localized proteins were randomly chosen from the predicted plastidial proteins (by TargetP) subtracted by those with membrane spanning domains (predicted by TMHMM) and known thylakoid proteins.

[b] Number of ESTs corresponding to the analyzed genes. The ESTs for thylakoid and stromal proteins were from TIGR Gene Index Tentative Consensus.

[c] Percent total number of genes analyzed that showed difference in EST abundance (at probability of difference being by chance less than 0.05 and 0.01) in pair wise comparisons between 'seeds', 'leaves', 'flowers', 'roots' library pools. The actual number of genes is parenthesized. Probability calculations were according to Audic and Claverie (1997).

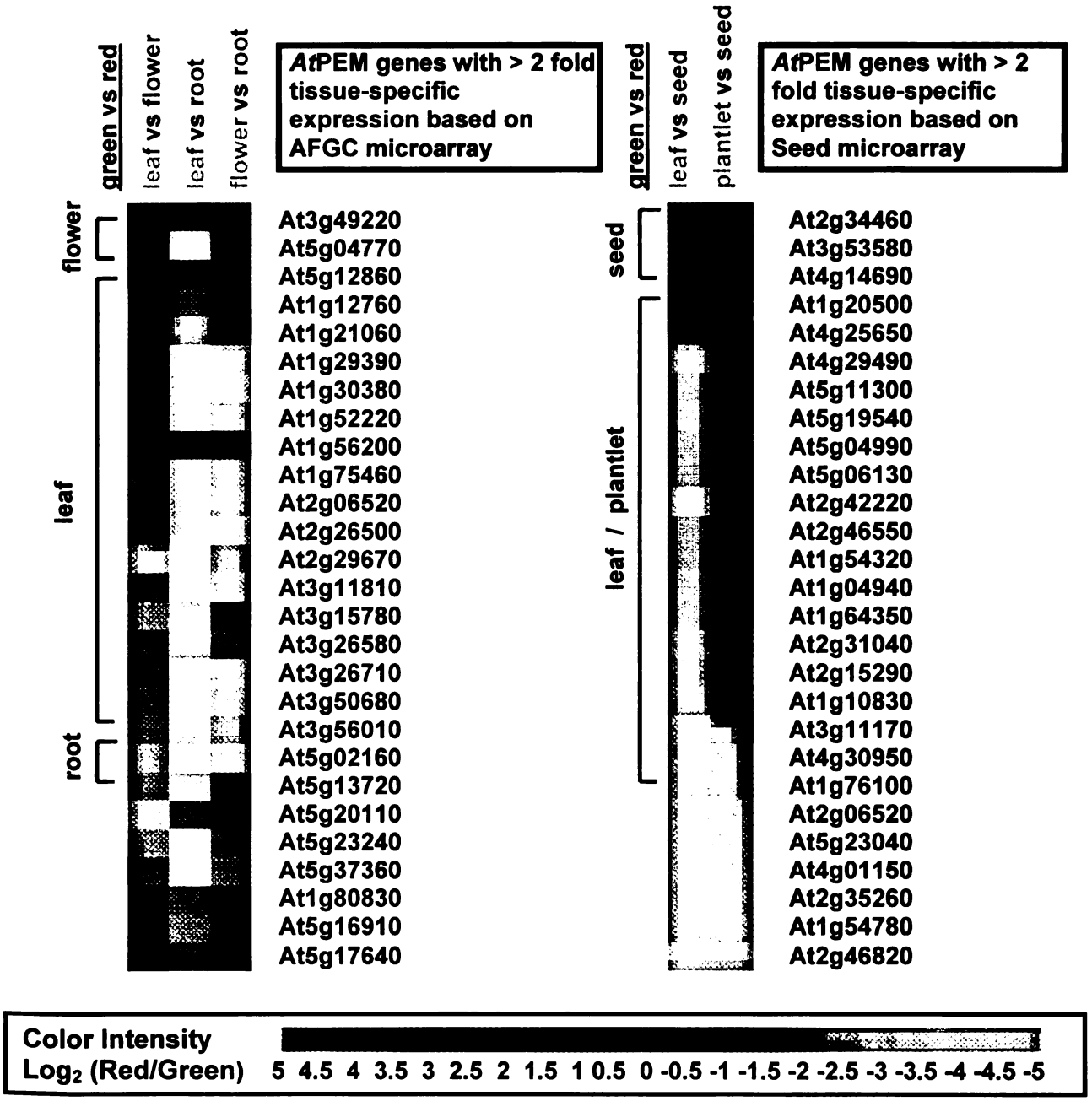
compared to that of root whereas only 3 showed higher levels in root than in leaf. Twenty one of these 22 candidates (expressed higher in leaf than in root) also had higher transcript abundance in leaf than that in flower whereas only 3 candidates showed higher expression in flower than in leaf. Comparison between the seed and the leaf also showed higher levels of transcript abundance in leaf (4 higher in seed, 23 higher in leaf). Although it is well known that thylakoid and stromal chloroplast proteins are very abundant in leaves, very little comparative information is available on the envelop proteins. The data of Figure 24 suggest that transcripts for many plastid envelope proteins are also more abundant in leaves than from heterotrophic tissues.

Six genes selected by the digital expression profile analysis in Table 10 as tissue-specific were also present in the microarray data shown in Figure 24. Although the digital expression profiles used mixed tissues as a reference and microarray data compared individual tissues or plantlets, data for all six genes agree at least partially between the two types of analysis. However the cross validity between the statistical EST analysis and the microarray analysis should be further characterized by larger sets of genes which have data analyzed by both methods, combined with additional experiments (northern blots, RT-PCR, etc.) to verify results.

Changes in transcript abundance in seeds during 5 to 13 days after flowering were recently reported by Ruuska et al. (2002). Twenty-six *At*PEM candidates changed more than two fold during this period (data not shown). The expression of many genes involved in seed storage protein, starch and lipid biosynthesis, also change during these

Figure 24. Tissue-specific microarray analysis of *At*PEM candidates.

*At*PEM candidates with > 2 fold tissue-specific expression in at least 2 different tissue comparisons are shown clustered according to the tissue specificity (indicated with the brackets and labeled). The intensity of green and red colors represents the relative level of transcripts (refer to the color keys at the bottom for the actual ratios) in tissues under comparison (the tissues under comparison are indicated above the image and colored with representative colors). The data for flower, leaf, root comparisons were from SMD (http://genome-www.stanford.edu/microarray) and data for seed versus leaf and seed versus plantlet were from the Arabidopsis developing seed array (Girke et al., 2000; Ruuska et al., 2002). The graphic was generated by Tree View software (Eisen et al., 1998).


green vs red
leaf vs flower
leaf vs root
flower vs root
AtPEM genes with > 2 fold tissue-specific expression based on AFGC microarray
flower
leaf
root
At3g49220
At5g04770
At5g12860
At1g12760
At1g21060
At1g29390
At1g30380
At1g52220
At1g56200
At1g75460
At2g06520
At2g26500
At2g29670
At3g11810
At3g15780
At3g26580
At3g26710
At3g50680
At3g56010
At5g02160
At5g13720
At5g20110
At5g23240
At5g37360
At1g80830
At5g16910
At5g17640
green vs red
leaf vs seed
plantlet vs seed
AtPEM genes with > 2 fold tissue-specific expression based on Seed microarray
seed
leaf / plantlet
At2g34460
At3g53580
At4g14690
At1g20500
At4g25650
At4g29490
At5g11300
At5g19540
At5g04990
At5g06130
At2g42220
At2g46550
At1g54320
At1g04940
At1g64350
At2g31040
At2g15290
At1g10830
At3g11170
At4g30950
At1g76100
At2g06520
At5g23040
At4g01150
At2g35260
At1g54780
At2g46820
Color Intensity
Log2 (Red/Green)
5 4.5 4 3.5 3 2.5 2 1.5 1 0.5 0 -0.5 -1 -1.5 -2 -2.5 -3 -3.5 -4 -4.5 -5

stages (Ruuska et al.,2002) and therefore these 26 *At*PEM candidates may be involved in the role of plastids in seed filling and development.

Towards the Function of Unknown Candidates

Gene knock-outs often can provide key information to link genes of unknown function to a phenotype. Large collections of T-DNA insertion mutants are publicly available and when the DNA flanking the T-DNA insertion sites are sequenced and aligned with the Arabidopsis genome sequence, provide mutants useful for studying gene function. We searched the Sequence-Indexed Library of Insertion Mutations generated by the Salk Institute Genome Analysis Laboratory (http://signal.salk.edu/tabout.html; containing 32,758 T-DNA sequences as of March 8, 2002). In total 388 insertion lines corresponding to 217 *At*PEM candidates (approximately 40% of *At*PEM candidates) were found. The

'Insertion IDs' can be used to search and to order the mutant line seed stocks from the Arabidopsis Biological Resource Center (ABRC,http://www.biosci.ohiostate.edu /~plantbio/Facilities/abrc/abrchome.htm) at Ohio State University. These 'Insertion IDs' as well as the accession numbers to search the publicly available microarray data in SMD (http://genome-www.stanford.edu/microarray) were deposited on our web site (http://www.plantbiology.msu.edu/PlastidEnvelope/).

Part II. Candidates for Lipid Transporters of the Plastid Envelope

Plastid targeted ABC transporters

The ATP-binding cassette (ABC) protein super family is one of the largest protein families known in the Arabidopsis genome with more than 120 members (Sanchez-Ferdandez et al., 2001). The overexpression of some ABC proteins has conferred multidrug resistance phenotypes in mammals (Gottesman and Pastan, 1993). Some of the known functions of ABC transporters include i) plasma membrane efflux pumps for the amphipathic cations, ii) translocation of cationic phospholipids between membrane bilayer leaflets, iii) glutathione S-conjugate pumps of organic anions, iv) transport of anticancer drugs, pigments, iron-sulfer complex, peptides and long-chain acyl-CoA substrates (See Sanchez-Fernandez et al., 2001 for review). Therefore we hypothesized that some of the ABC transporters in Arabidopsis might be involved in the hydrophobic/amphipathic lipid movements across the plastid double-membrane envelope.

There were 16 potential ABC transporters among the *At*PEM candidates (Table 7). In order to include as many candidates as possible for the reverse genetics screening, 4 additional ABC transporters (At5g61700, At3g47750, At5g61690 and At5g61740) were included as potential ABC transporters in the plastid envelope. Target P predicted those 4 proteins as mitochondrial targeted whereas another subcellular localization predictor, Predator (http://www.inra.fr/predotar/) predicted them to be either dual targeted or plastidial. Among these 20 candidates, T-DNA insertion mutant lines were available for 11 candidates from the Salk Institute Genome Analysis Laboratory (http://signal.salk.edu/tabout.html, Table 12). The T-DNA (5.5-kb) insertions into these 11 candidates were identified by PCR screening. Homozygous insertion lines for 9 candidates were established which showed no PCR bands with gene specific primers but showed bands

with a gene specific primer and a T-DNA left border primer set. The identities of the amplified bands were confirmed by sequencing for some of those lines (SALK_013945, SALK_000578, SALK_015438, SALK_016209) (data not shown). No homozygous knock-outs were found from the seed stocks SALK_008761 (At1g15210) and SALK_ 056267 (At3g25620) (Table 12).

Phenotypic Observations and the Fatty Acid Composition/Contents in the KO plants of the Plastid ABC transporter candidates

What would be the expected phenotype if the lipid transporters on the plastid envelope are disrupted? Many Arabidopsis lipid mutants do not display dramatic growth phenotypes because Arabidopsis possesses two different pathways for the synthesis of membrane glycerolipids, namely, prokaryotic and eukaryotic pathways (Browse and Somerville, 1994). When one pathway is blocked, the cell redirects fatty acid flux through the other pathway to compensate the losses. For example, *act1* mutants have a defect in the acyl-ACP sn-glycerol-3-phosphate acyltransferase, the first enzyme of the prokaryotic pathway but the plant do not look different from the wild type plants because the deficiency is compensated for by increase in the eukaryotic synthesis of chloroplast lipids (Kunst et al., 1988). Partial deficiency in the export of certain fatty acids resulted in reduced growth and abnormality in seeds in plants knocked out in the *FATB* (acyl-acyl carrier protein thioesterase B) gene (Bonaventure et al., 2003). However, the major cause for the phenotype was attributed to the lack of palmitate not the reduced amount of the fatty acid exported from the chloroplast. If fatty acid export from the plastid is mediated

Table 12. The Arabidopsis T-DNA insertion mutants for the predicted plastid ABC transporters.

Name	Locus ID	[a] Stock ID	[b] insertion	Gene specific primers for PCR screen
ID 1	At1g15210	SALK_008761	Exon	5'-CGTGTGGAACAGGTTACCTC-3' 5'-CTGGTGATGAGGACTTACGG-3'
ID 2	**At1g15520**	**SALK_013945**	Exon	5'-GTTCCAGGTTTTCATCGAGA-3' 5'-TATTCTGTGGACATAGCGAGAG-3'
ID 5	**At1g59870**	**SALK_000578**	Exon	5'-CTAAATGGGGTCATCGTTATTG-3' 5'-CCATAACGAGTACCAACACCTT-3'
ID 11	**At1g70610**	**SALK_015438**	Intron	5'-TGGATGTCAAGTGGCTTAGG-3' 5'-TGGAGAGTGAGGGTGATGAA-3'
ID 13	**At2g39190**	**SALK_016209**	Intron	5'-CCACTGTAGGTGAACAAAGGG-3' 5'-ACCGAATGAAGAATCTTCCTTG-3'
ID 21	At3g25620	SALK_056267	Exon	5'-CGCGTTGCTCATTTACAAGA-3' 5'-CCACAACAAAGCCACTCATG-3'
ID 23	**At5g03910**	**SALK_052673**	Exon	5'-TGGAAAAAGTTGCTGGAGAAG-3' 5'-TCAAAAAGGCATAACTAGGGAA-3'
ID 27	**At5g64940**	**SALK_045739**	Intron	5'-CTTGATCCTTGTACCCTTGC-3' 5'-TAGTTGCAGGTGTCTGAGAACA-3'
ID 32	**At3g47750**	**SALK_041452**	L300-5'	5'-TGGTTTACCTCGTTATCTTCGT-3' 5'-TGGGAAGATCTGAGTGAATCC-3'
ID 26	**At5g52860**	**SALK_015052**	Exon	5'-CCACACAAGACTCGCTCAAG-3' 5'-GGCAAGAGAGCTCAAGAAGAG-3'
ID 71	**At1g54350**	**SALK_003891**	Exon	5'-GAGTCTCAGCAGCGTGATGTT-3' 5'-GAGATAGCGCTGCAGGTAAT-3'
		SALK_069087	Exon	5'-GAGTCTCAGCAGCGTGATGTT-3' 5'-ATCACACTGCTTAACGAGGC-3'

Note. The lines with homozygous T-DNA insertion found were indicated by bold cases.

[a] seed stock identification numbers from the Arabidopsis Gene Mapping Tool (http:// signal.salk.edu/cgi-bin/tdnaexpress) and from the Arabidopsis Biological Resource Center.

[b] the position of T-DNA insertion from the Arabidopsis Gene Mapping Tool.

by proteins then disruption of such proteins may cause increased flux through the prokaryotic pathway which may result in increased 16 carbon fatty acids in the overall fatty acid composition. Alternatively, growth impairment might occur if free fatty acids were to accumulate or if the defect reduces the export of the saturated or other fatty acids which play a crucial role for plant growth. It may also be possible to detect the effect of blockage in the return of lipids from the ER pathway through fatty acid composition analysis by examining the signatures typical for each pathways (lipids derived from the prokaryotic pathway contain 18 carbon fatty acids at the sn-1 position and 16 carbon fatty acids at the sn-2 position whereas ER derived lipids contain 18 carbon fatty acids in both positions). A mutation in the recently discovered permease-like protein (*TGD1*), which was proposed to be involved in the transfer of diacylglycerol moiety from the ER to the plastid, showed increased prokaryotic pathway-derived galactolipids (Xu et al., 2003). This mutation however did not cause decrease in the overall amount of galactolipids nor did it show significant growth phenotype, again showing the remarkable flexibility of the plant using the alternative pathways. The enzymes responsible for the MGDG and the DGDG synthesis (*MGD1* and *DGD1* respectively) are localized specifically to the inner membrane and to the outer membrane of the plastid envelope (Awai et al., 2001; Froehlich et al., 2001). The MGDG synthesized in the inner membrane needs to be transported to the outer membrane for DGDG synthesis. The synthesized DGDG should be transported back to the inner membrane and eventually transferred to the thylakoid membrane. A more drastic effect is expected similar to that in the *dgd1* mutant if any of these processes are disrupted for DGDG availability in the photosynthetic membrane.

None of the selected 9 homozygous insertion mutants showed any visual growth phenotype. Initially, when the 132 (11 candidates × 12 plants per each candidate) T3 seeds from ABRC stock center were grown, many showed various abnormalities including slow growth, very late bolting, smaller plants etc. However, these phenotypes did not correlate well with the homozygosity of the insertion events. Furthermore, when seeds collected from these plants were re-grown (next generation), most of the abnormal phenotypes were abolished. The fatty acid composition of leaves and seeds of the 9 homozygous T-DNA knock-out plants are shown in Table 13. When compared to the samples from the wild-type plants there was no marked change in the fatty acid compositions. The oil content in the seeds was also analyzed and lines that showed marked changes are presented in Table 14. The homozygous knock-outs of At1g70610 and At5g64940 displayed reduction in the seed oil contents (60-70% of the wild type seed oil content). When the seeds from the progeny (T4 generation) of these mutants were analyzed, they consistently showed reduced oil contents in seeds (ID 11-3-1-2, ID 27-8-1-1, ID 27-8-1-3 in Table 14). These results are still preliminary (because the purpose of this initial analysis was to select more interesting lines among the large number of plants) and needs more accurate statistical measurements to confirm the results.

CONCLUSIONS

In this study we attempted to predict integral plastid envelope proteins from the *Arabidopsis thaliana* nuclear genome using computational methods (Part I) and from these candidates, putative plastidial ABC transporters were selected and studied by reverse genetics approach in an effort to identify proteins that could potentially be involved in the lipid transport across the envelope membranes (Part II). So far there has been no established inventory of plastid envelope proteins. This work is one of the first published (Koo and Ohlrogge, 2002) works attempting to create a database for the entire plastid envelope proteome from *Arabidopsis thaliana*.

As the word 'candidates' implies the results of our study are not definitive due to the nature of prediction software and due to the possibility of errors in protein annotations in the genome databases (van Wijk, 2001). Although it is likely that at least 10% of the *At*PEM candidates represent incorrect predictions, it is reasonable to assume that the selected candidates represent a large portion of the real envelope proteome.

In addition to a broader interest in plastid metabolism, my specific interest was to identify putative lipid transporters in the plastid envelope. The road to the actual identification and characterization of a novel lipid transporter by these approaches is still long. However, the initiated analysis of putative ABC transporters of the plastid membrane lays a foundation towards future attempts to include all the possible ABC transporter

Table 13. Percent wild type fatty acid compositions in the leaves and seeds of the homozygous T-DNA knock-out plants of the plastid ABC transporter candidates.

	leaves		seeds			
FAME class [a]	C16	C18	C16	C18	C20	C22
ID 2-1	96.4	101.8	93.3	92.3	93.2	104.2
ID 2-1-1	-	-	110.2	105.4	86.9	82.7
ID 5-3	93.9	103.8	102.8	102.4	106.5	95.6
ID 11 [b]	96.1	102.0	96.6	96.3	98.5	101.6
ID 11-3-1-2	-	-	114.9	108.1	85.8	-
ID 13-5	100.0	99.6	101.5	102.0	107.0	95.0
ID 23-5	89.6	105.5	99.5	98.5	102.1	105.6
ID 26 [b]	-	-	99.8	100.6	97.9	100.6
ID 27 [b]	92.1	104.3	94.6	94.6	99.6	112.9
ID 27-8	-	-	109.3	106.2	87.4	44.9
ID 32 [b]	91.1	107.3	95.1	97.7	103.0	104.3
ID 43-3	-	-	97.3	92.6	89.9	108.3
ID 43-3-1-1	-	-	104.3	100.9	97.4	97.3
ID 71 [b]	-	-	103.5	100.7	98.0	100.4

Note. The name of each knock-out lines follows Table 12. Tissue samples from 3-5 independent wild type plants were used for the composition analyses and the average values are uses as references.

[a] fatty acid methyl ester species in the total lipid extract classified according to the number of carbons.

[b] values of these lines are average values of samples from at least 3 independent progenies.

Table 14. Homozygous knock-outs of plastid ABC transporter candidates that showed reduced oil content in seeds compared to the wild type plants.

Locus ID	Name	Homozygous KO lines	oil content (μg/20 seeds)	% wild type[a] oil content
At1g70610	ID 11-3	SALK_015438-3	66.5	55.17[b]
	ID 11-5	SALK_015438-5	71.3	59.16[b]
	ID 11-3-1-2	SALK_015438-3-1-2	66.3	54.83
At5g64940	ID 27-8	SALK_045739-8	89.1	73.95[b]
	ID 27-8-1-1	SALK_045739-8-1-1	72.4	59.86
	ID 27-8-1-3	SALK_045739-8-1-3	71.9	59.49

[a] Oil contents of seeds from 3-5 independent wild type plants were used as references.

[b] average of 2 independent extractions.

candidates (only roughly half were analyzed so far) in the search for lipid transporters and towards the bigger plan of assigning function to all genes of Arabidopsis.

MATERIALS AND METHODS

Establishment of a Database for Plastid Envelope Protein Candidates

The results of TargetP prediction for Arabidopsis chromosome 2 and chromosome 4 were from the web server at http://www.cbs.dtu.dk/services/TargetP/predictions/pred.html. The sequences for these two chromosomes were from The Institution of Genomic Research (TIGR) and the European Union Arabidopsis Sequencing Consortium (as of January 7, 2000). Additional sequence retrieval was from the National Center for Biotechnology Information (NCBI) Batch Entrez web-server (http://www.ncbi.nlm.nih.gov:80/Entrez/batch.html). Chromosome 1, 3, and 5 sequences were downloaded from the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis thaliana* Database ftp site (ftp://ftpmips.gsf.de/cress/, as of June 2001). Subcellular localization was predicted using TargetP v1.01 from the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/TargetP/). No cutoff was applied but instead 'Reliability Class' (RC) values from 1 to 5 were designated for each predicted proteins. Proteins with plastid transit peptides were then evaluated for membrane spanning domains using the TMHMM v2.0 web server at http://www.cbs.dtu.dk/services/TMHMM-2.0/.

Proteins that were not strongly predicted to be transmembrane proteins by the program yet contained weak α-helical domains with probabilities over 50% were also included in the database as possible membrane proteins. Proteins with transmembrane α-helical domains within the range of possible plastid targeting sequences (plastid targeting sequence prediction was according to the TargetP cleavage site prediction) were removed and in cases where the domain located close to but not within the predicted cleavage site were marked 'N-term' in order to reduce false prediction of hydrophobic targeting sequences as membrane spanning domain. Proteins that are known to locate in thylakoid were removed manually.

Classification by Function

Functional classification was based on the MIPS *Arabidopsis thaliana* Database automatically derived functional categories. The catalogue was downloaded from the Protein Extraction Description and Analysis Tool (PEDANT) web server (http://pedant.gsf.de/). The catalogue of genes for plant glycerolipid biosynthesis was from the web server at http://www.canr.msu.edu//lgc/ (Mekhedov et al., 2000). An updated inventory of lipid metabolism related genes was available at our regional database (Beisson et al., 2003). The full inventory of Arabidopsis ABC proteins was downloaded from the website at http://www.arabidopsisabc.net/ (Sanchez-Fernandez et al., 2001) and was queried for *At*PEM candidates.

Digital mRNA Expression Profiling

A set of all public Arabidopsis ESTs was obtained through a Structured Query Language (SQL) query of our in house 'SeqStore' database which contained 103,109 EST sequences from GenBank Expressed Sequence Tag database (dbEST, http://www.ncbi.nlm.nih.gov/dbEST/index.html). The EST sequences were used as queries (BLASTN version 2.2.1) against the target database of all predicted transcripts from Arabidopsis genome (ftp://ftp.tigr.org/pub/data/a_thaliana/ath1/, January 10, 2002). Then the plastid envelope protein candidates were queried against this resulting database using common chromosomal locus identifiers (i.e. At2g39040). Tentative Consensus (TC) sequences assembled from ESTs and Expressed Transcript (ET) sequences were retrieved from TIGR Arabidopsis Gene Index (http://www.tigr.org/tdb/agi/). In order to match TCs with the chromosomal locus identifiers, TCs retrieved were mapped to all the predicted transcripts of Arabidopsis genome by Blast alignments (BLASTN version 2.2.1). TCs corresponding to the plastid envelope protein candidates were selected by chromosomal locus identifiers.

To identify possible differential expression of the *At*PEM candidates, the relative frequencies of ESTs between tissue-specific library pools were compared (55 different EST libraries were grouped into 8 tissue–specific 'library pools' according to the source tissues from which the library was derived (Beisson et al., 2003). The influence of random fluctuations and sampling size was considered statistically to discern the reliability of the digital expression profiling (Audic and Claverie, 1997). For this analysis, software was downloaded from http://igs-server.cnrs-mrs.fr. EST analysis for

the thylakoid and stromal proteins was done using 39 selected known thylakoid localized proteins, most of which are related to photosynthesis, and 1,802 stromal proteins which were chosen randomly from plastidial proteins predicted by TargetP and subtracting those with transmembrane domains and obvious thylakoid proteins. ESTs for the thylakoid and stroma localized proteins were from TIGR Arabidopsis Gene Index TCs.

Microarray Data

Public microarray data for *At*PEM candidates were searched from a local database that contained most of the microarray data downloaded from Stanford Microarray Database (as of March 1, 2002, Sherlock et al., 2001,) and was courtesy of Rodrigo Gutierrez. The duplicates in the tissue comparison data sets (SMD experiment identifiers: 7197, 7199, 7200, 7201, 7203, 7205) were averaged and divided each other in a way to give pair wise comparisons between flower, leaf and root tissues. Arabidopsis developing seed array data was from local database and are available at http://www.bpp.msu.edu/Seed/SeedArray.htm (Girke et al., 2000; Ruuska et al., 2002). The cluster image (Figure 24) was generated by Tree View software downloaded from the website at http://rana.lbl.gov/ (Eisen et al., 1998).

T-DNA Insertion Mutants

T-DNA insertion mutant lines were searched from the Sequence-Indexed Library of Insertion Mutations generated by the Salk Institute Genome Analysis Laboratory using Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress).

Information on *At*PEM candidates and their various attributes including tissue-specific EST frequencies, accession numbers to search SMD, T-DNA insertion mutant stock numbers from Salk Institute Genome Analysis Laboratory can be downloaded from our web site at http://www.plantbiology.msu.edu/PlastidEnvelope/.

Homozygous T-DNA Insertion Mutant Isolation

T-DNA insertion mutant lines for plastid ABC transporters were searched by Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) and the mutant seed stocks were obtained through the Arabidopsis Biological Resource Center (ABRC, http://www.biosci.ohiostate.edu/~plantbio/Facilities/abrc/abrchome.htm) at Ohio State University. Homozygous KO lines were selected among the T3 generations by PCR screening for both the presence of T-DNA insertion and the absence of an intact gene. The gene-specific primers used for the screening of insertions are shown in Table 12. The T-DNA–specific primer matching the left end of the T-DNA was 5' TGGTTCACGT AGTGGGCCATCG 3'. Seeds from the selected homozygous knock-outs (T4 generation) were grown again and the second round screening was carried out to confirm the homozygosity.

Fatty Acid Composition and Content Measurements by Gas Chromatography

About 0.1 g fresh weight leaves or 20-30 seeds were heated at 90°C for 1h 30 min in 0.3 mL of toluene and 1 mL of 5% (v/v) H_2SO_4 with heptadecanoic acid (17:0) as an internal

standard. After the samples were cool, 1.5 mL 0.9% (w/v) NaCl was added and fatty acid methyl esters were extracted 3 times with 2 mL of hexane. Samples were evaporated under a stream of N_2 and redissolved in 300 μL hexane. The fatty acid methyl esters were analyzed by GC with a flame ionization detector (GC-FID) on a DB-23 capillary column (J&W Scientific, Folsom, CA).

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CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH PERSPECTIVES

Understanding how free fatty acids are exported from the plastid has been one long-standing missing link in basic knowledge about plant lipid biochemistry. In most reviews of plant lipid metabolism the export process has usually been drawn on biochemical pathway diagrams without much elaboration as a continuous arrow from the site of *de novo* fatty acid synthesis (FAS), the stroma, to the endoplasmic reticulum (ER) membranes. In my work on fatty acid export presented in chapter II of this thesis (also published in Koo et al., 2004), a new focus was applied to FA export and the process was discussed in much more details, raising questions that were not so deeply asked in the past. The data in Chapter II define and measure parameters that are needed to better understand the molecular mechanisms of fatty acid export from the plastid in plants. The data have suggested a more complicated mechanism than just a simple physical diffusion model where the free fatty acid (FFA) flip-flops across the inner envelope membrane, dissociates into the intermembrane space and finally repartitions into the outer envelope membrane where the long-chain acyl-CoA synthetase (LACS) captures the FFA and thus drives the net flow of the FFA from the stroma to the cytosol. A major conclusion of Chapter II is that the data discount this simple free diffusion-based model for FFA export from the plastid.

Three models for the mechanism of fatty acid export from the plastid and directions for future studies

Three generic models (and combinations thereof) can be proposed as alternatives to the simple diffusion model and are based on our current knowledge about the plastid envelope and the enzymes related to the fatty acid metabolism. These models are: (1) a non-protein-mediated facilitated-diffusion (channeling) between the stromal acyl-ACP thioesterase and the outer envelope long-chain acyl-CoA synthase, (2) a cryptic inner envelope LACS, and (3) intervening transfer proteins. These models are explained below together with suggested directions for future studies to characterize and discriminate between them.

Non-protein-mediated facilitated-diffusion begins with the preferential partitioning or loading of newly synthesized free fatty acid onto the inner envelope membrane. The exact sub-plastidial organization of the members of the FAS enzymes *in vivo* is not known although some researchers believe it to be formed into a supra-molecular complex in the stroma. The individual FAS enzymes are soluble enzymes but the observation that the plastid retains FAS activity even when the isolated plastid is swollen to release up to 50% of its soluble proteins into the extraplastidial media make us believe that they are formed into an enzyme complex (Roughan and Ohlrogge, 1996). The multisubunit acetyl-CoA carboxylase is found to be strongly associated with the chloroplast envelope through non-ionic interactions which supports the FAS super-complex association with the envelope (Thelen and Ohlrogge, 2002). The final enzyme in the series of fatty-acyl

chain elongation reaction of FAS before the export from the plastid is acyl-ACP thioesterase. The acyl-ACP thioesterase hydrolyses the growing chain of fatty acids from acyl carrier proteins. Unpublished studies suggest at least the FatB form of this enzyme is associated with the envelope membrane when imported into the pea chloroplast by *in vitro* reconstitution experiments (communicated by Dr. John Froehlich, MSU-DOE Plant Research Laboratory). The acyl-ACP thioesterase association with the inner membrane should be characterized more carefully since it can partly explain the uni-directional movement of the free fatty acid towards exit from the plastid by loading the FFA directly on to the membrane.

Second, further studies about the plastid envelope localized long-chain acyl-CoA synthesis (LACS) enzymes are critical to gaining insights into the mechanism of fatty acid export for all 3 models. From the data (chapter II) we learned that LACS has a) excess capacity (may run at least 10 times faster than the *de novo* FAS rate) to handle the entire flux of free fatty acid coming out from the site of synthesis, b) has very low *in vivo* K_m (the long-chain acyl-CoA production reached steady state at very low amount (bulk concentration) of free fatty acid), and c) also has priority for binding FFA over the extraplastidial proteins such as BSA. If the LACS can access FFA substrates supplied directly from the inner membrane then at least most of our observations regarding the efficient *in vivo* pool ('pool A') can be explained without invoking other transport proteins. At this point it is not clear how many or which are the major players of envelope LACS that is/are involved in fatty acid export. `There are 4 candidate genes for the plastid LACS proteins in the Arabidopsis genome. The null mutants of these genes in

combination with localization studies should identify the major LACS's. So far knock-out plants for the suggested major plastid envelope LACS gene showed no growth phenotype nor was there any compositional change in the lipids that might suggest compromised export of acyl groups from the chloroplasts (Schnurr et al., 2002). In fact the single T-DNA insertion knock-outs for all 4 candidates displayed no visible phenotypes (data not shown) making it very important to create multiple knock-outs. According to the current two-pathway scheme for the glycerolipid synthesis, disruption of all envelope LACS proteins should cause major problems in the synthesis of ER derived lipids and theoretically be lethal unless the other pathway can compensate for the loss. A similar phenotype could be expected from mutants that are blocked in the fatty acid export pathway from the plastid (for example, by knock-out of all acyl-ACP thioesterase genes).

Once a LACS involved in fatty acid export is identified, experiments to find out the precise location of the protein, whether on the inner envelope membrane facing the intermembrane space or on the outer envelope facing the intermembrane space/cytosol, should be pursued. This will provide important clues for the non-protein-mediated facilitated diffusion model and the 'cryptic' LACS model. Protease protection assays with either the *in vitro* plastid-targeted radiolabeled-LACS protein or the chloroplasts with endogenous LACS using western blot as a detection method can be used (antibody specific for this LACS should be raised and since LACS genes are not very well conserved in sequence this shouldn't be difficult). In addition using protease in combination with the kinetic analysis of acyl-CoA synthesis with the isolated

chloroplasts may also give useful information about the contribution by different LACS's. However, if these multiple knock-outs show no effect on the ER-pathway, there will need to be a paradigm change in our current scheme about fatty acid delivery from the plastid to the ER and about the involvement of the known plastid LACS's in this process.

Third, several different approaches were/can be taken in order to test if a protein mediated transport mechanism (model 3) is involved in FA export. However, these approaches require more knowledge about the fatty acid export to be accumulated before taking major risks, such as forward genetic screening or biochemical purification, to identify the protein component of the export system. In this thesis I tried to lay the foundations on which the future works can be built upon. As described briefly in chapter 1, I first began to collect information about lipid transporter candidates that have been identified in various other organisms. Through this study I learned that at least the long-chain acyl-CoA synthetase family and the ABC transporter family were consistently involved in various aspects of lipid transport. This led me to focus on these proteins in Arabidopsis. Eventually, as more and more lipid transporters get to be identified, the sequence information from these proteins can be used to identify common lipid binding motifs. This is especially important because according to my blast search attempts of the animal- or bacterial-lipid proteins against the Arabidopsis genome sequences, the majority did not have obvious orthologs in Arabidopsis. Making an inventory of this review work about the identified lipid transporter candidates in all organisms should help

to organize our efforts in the future. As far as I know there is no such gene catalog available yet that focuses on lipid transport.

Next I again took a more general approach to increase our understanding about the plastid envelope proteome using bioinformatics tools. The plastid envelope is the site of various biochemical reactions, including many of lipid metabolism and also serves as the interface/barrier for many different cellular metabolisms which should be coordinated. Since membrane proteins are harder to study, much less was known about the hydrophobic integral membrane proteins of the envelope (only 34% of the *At*PEM candidates could be assigned to putative functions). The completed genome sequence of Arabidopsis opened up a new opportunity to tackle this problem. I used various computational methods to predict the integral plastid envelope membrane proteins. Other information such as number of deduced amino acids, number of transmembrane domains, expected cleavage sites of presequences, putative functions, number of expressed sequence tags (EST's) and their source libraries, microarray data, and the publicly available T-DNA insertion mutants, etc., were combined and were made into an internet based data base (*At*PEM) available for the public (http://www.plantbiology.msu.edu/PlastidEnvelope/index.htm). The *At*PEM became one of the first published databases for plastid envelope membrane proteins (Koo and Ohlrogge, 2002).

A reverse genetics approach was applied next to search for lipid transporters among the *At*PEM candidates. The approach was designed not only to look for proteins involved in FFA export but also to look for proteins involved in the import of the ER pathway derived lipids. The disruption in one of the two glycerolipid synthetic pathways is

expected to accompany the changes in the fatty acid composition as was exemplified by many mutants in these pathways (Browse and Somerville, 1994). The *tgd1* mutant, which was implied to be disrupted in the transfer of diacylglycerol moiety from the ER to the plastid, also showed changes in the fatty acid composition, i.e., increase in the prokaryotic pathway-derived galactolipids (Xu et al., 2003). Twenty ABC transporter family member genes were selected as such candidates and 11 available T-DNA insertion mutants were obtained. Among these 11, homozygous insertion mutants were established for 9 of them. These were screened for visual phenotypic changes and for fatty acid composition/content changes. In the initial screening of fatty acid composition, changes were not observed, which might have been expected for the disruption in one of the two ways of glycerolipid synthesis. However, two knockout lines were found to have reduced oil content in their seeds. This result must be verified through statistical analyses to evaluate the significance of the changes and also through complementation experiment. In addition the time course of the oil accumulation during seed development should give more convincing results concerning abnormality in the oil content. In order to understand whether the effect of the gene disruption was related to the influence on lipid metabolism, fatty acid (pulse-) labeling experiments should be conducted. Ultimately the transgenic plants over-expressing these genes can be tested to see their ability to bring changes in oil content in seeds.

In addition, only 11 of 20 of the putative plastid ABC transporters have been examined and the other 9 should be considered when the T-DNA knock-outs become available. There are some additional characteristics to be examined with all the candidate knock-

outs in addition to the changes in fatty acid compositions and oil contents. First, the disruption in FA export may result in increased FFA export pool size. Short pulse labeling of FA's by radiolabeled acetate could help to measure the changes in the FFA pool size among those mutants. Second, the mutants with decreased DGDG contents and increased MGDG contents could potentially identify a MGDG translocator between the inner membrane and the outer membrane. The mutant plant with this type of lipid composition is expected to show a visible growth phenotype similar to that observed in the *dgd1* mutant. Perhaps the involvement of proteins in this process can be tested in a separate experiment using various inhibitors of the ATPases, the P-glycoproteins and the multidrug resistance proteins that are implied to be involved in lipid transport (i.e. vanadate, verapamil, colchicines and vinblastine). Simple assays can be developed first to test the effects of these drugs on the synthesis of DGDG from the radiolabeled UDP-galactose. If the MGDG synthesis is not affected yet the DGDG synthesis gets inhibited by those drugs this may indicate the involvement of those drug-sensitive proteins in the translocation of MGDG from the inner membrane to the outer membrane. However the indirect effects of those drugs on other pathways in plants should be taken into account.

If a putative glycerolipid transporter is identified through the T-DNA knock-out strategy, then *in vitro* lipid transport assays in artificial-membrane vesicles can be used to directly demonstrate its function. One example of such assays was developed by Stephan Ruetz and Philippe Gros (1994) and was used to demonstrate the function of the Mdr2 protein as a PC translocase. First, in order to prepare the artificial-membrane vesicles with the expressed protein in same polarity (the expressed protein in these vesicles all face

outside), a yeast mutant, defective in the last step of the vesicular fusion pathway was used. This mutant accumulates large amounts of unfused secretory vesicles at the nonpermissive temperature. Then they used the internalization of the fluorescent PC analog into the artificial-membrane vesicles in the presence/absence of the expressed Mdr2 protein to demonstrate its function as a PC translocase. The asymmetric distribution of the fluorescent PC in the outer and the inner leaflets of the lipid bilayer was measured by fluorescence spectroscopy. Following the addition of a membrane impermeable, fluorescent-PC reducing agent (dithionite) the internalized fluorescent PC was deduced from the residual fluorescence. Similar assay have been used in plants by Gomes et al.(2000) to demonstrate the role of an Arabidopsis P-type ATPase, *ALA1,* in the internalization of phosphatidylserine in reconstituted yeast membrane vesicles. These assays in combination with the use of ATP, Mg^{2+} and various inhibitors of ABC transporter will provide more direct evidence of the putative protein being a genuine phospholipid transporter.

One possible biochemical strategy to look for potential fatty acid transporters would use antibodies to proteins such as the acyl-ACP thioesterases or the LACS's. The protein cross-linking reagents can be used to cross-link the interacting proteins with the acyl-ACP thioesterases or the LACS's (Akita et al., 1997; Chou et al., 2003; Snyder et al., 2000). Following solublization to remove membrane lipids, the cross-linked protein complex can be immuno-precipitated with the antibodies raised against the acyl-ACP thioestrases or the LACS's. Alternatively, the cross-linked protein complex can be separated on a native polyacrylamide gel and detected by the western blot. Protein mass-

spectrometry can be used to identify the peptides in the complex. Antibodies raised against newly identified polypeptides can be tested for their ability to inhibit fatty acid uptake. In addition, the expression pattern of such proteins in those plant tissues that demand high fatty acid export from the plastid such as, in the rapidly expanding leaves or in the developing seeds at the stage of oil accumulation, may provide additional evidences that the protein is involved in fatty acid transport. At this point we don't have clear speculations about the effect of disrupting such components in plant. But perhaps the reagents that were used in the animal systems to block FA transport such as various anion transporter inhibitors, fatty acid analogues, photoaffinity labeling reagents, etc. (Abumrad et al., 1998), could be tried in isolated chloroplasts first for their effect on the fatty acid export. This type of inhibition studies although no guarantee for the success may give us information about the effects of disrupting fatty acid export from the plastid.

A novel fatty acyl activating enzyme in the plastid

In chapter III the plastid was demonstrated to be the major site for the elongation of the exogenous MCFA's into the C16 and C18 FA's by i) measuring the saturated to the unsaturated fatty acid ratio among the labeled products in the leaf fed with the radiolabeled MCFA's, ii) using inhibitors of fatty acid synthesis in the plastid and in the ER, iii) testing the ability of isolated chloroplast to elongate the exogenous MCFA and iv) using a mutant plant impaired in an acyl activating gene predicted to be targeted to the plastid. The fatty acid synthesis pathway in the mutant plant was intact, as demonstrated by using acetate as substrate for fatty acid synthesis, but the mutant plant was unable to

elongate the exogenous short- and medium- chain fatty acids. The gene (*AAE15*) disrupted in the mutant has sequence similarity to other known long-chain acyl-CoA synthetases and in fact was shown previously by another group to have no LACS activity in the heterologous expression systems. Measurement of the acyl-ACP and the acyl-CoA intermediate levels raises the possibility that the *AAE15* gene product is an acyl-ACP synthetase (AAS), an enzyme that catalyzes the direct esterification of the free fatty acid to an acyl carrier protein (ACP). The acyl-ACP synthetases (EC 6.2.1.20) have been isolated from bacteria and yeast (Rock and Cronan Jr, 1981; Fice et al., 1993; Jackowski et al., 1994; Gangar et al., 2001) but have not yet been demonstrated in plants. In my most recent assays using isolated pea chloroplast homogenates to test for *in vitro* AAS activity, an exogenous ACP-dependent acyl-ACP formation was observed (data not presented) further supporting the existence of AAS activity in the plastid. Similar assays should be carried out with chloroplasts isolated from both the wild type and the *aae15* knock-out Arabidopsis to relate the observed *in vitro* AAS activity with the *AAE15* enzyme. More directly the recombinant *AAE15*, expressed/purified heterologously in *E. coli* and in the yeast, should be tested for its ability to catalyze the *in vitro* AAS reaction. If the heterologous system gives positive results then more detailed enzyme characterization, including the substrate specificity test, should be followed.

The focus of future experiments on this topic should be to reveal the *in vivo* function of *AAE15*. The *aae15* knock-out plant is not visibly distinguishable from the wild type plant under normal growth conditions. However more detailed characterization of this mutant should be carried out both under normal conditions and stress conditions. Nine

out of 11 EST's of *AAE15* are derived from libraries that were constructed after dehydration and the cold treatments. The gene expression pattern of *AAE15* in the wild type plant and the phenotypic analyses of the *aae15* plants in these conditions may give us clues to the *in vivo* function. In addition the tissue specific expression pattern of *AAE15* should guide us where to focus in these analyses. One of the hypothetical functions of *AAE15* proposed in the discussion of chapter III was involvement in membrane editing and/or a proof reading function of *de novo* FAS. It is possible that some of the growing chain of fatty acids during FAS get released prematurely. In addition there are many uncharacterized lipase gene homologs in the Arabidopsis genome (Beisson et al., 2003) which could potentially be involved in the release of free fatty acids. It is possible that some of those lipases may become active under certain occasions, i.e. abiotic stresses, mechanical wounding, herbivor attacks, senescence, etc. to release free fatty acids. Perhaps plant cells use *AAE15* in a mechanism to recapture/recycle those released free fatty acid into the regular glycerol lipid synthesis. To test this hypothesis the fatty acid compositional changes in the *aae15*- ko plants in various conditions should be analyzed by GC and/or GC-MS. In addition it would be interesting to examine whether there is any change of the *AAE15* expression in the medium-chain acyl-ACP thioesterase-overexpressing plants which releases large amount of medium-chain fatty acids prematurely (Eccleston and Ohlrogge, 1998)

Perhaps the acyl-ACP formation and the elongation of exogenous free fatty acid by the isolated chloroplasts can be used to conduct *in vitro* kinetic analyses to study the import process. The utilization of the exogenous fatty acids was inversely proportional to the

fatty acid chain length and thus the long-chain fatty acids were poor substrates for the elongation by isolated chloroplasts. This was not due to substrate depletion by prior usage in the acyltransferase reactions before reaching the stroma, since no exogenous coenzyme A was provided in the assay and the majority of the long-chain fatty acid substrates stayed in free form at the end of the assay. Therefore possibly the substrate specificity of the pea/spinach homolog of *AAE15* for shorter fatty acids determines the relative ability of fatty acids to be elongated. However if it turns out that the *AAE15* gene product does have activity towards long-chain fatty acids such as palmitate then we could hypothesize that the import of the long-chain fatty acid was the problem. The free diffusion of the short- and the medium-chain fatty acids across the membrane seems to be very fast and thus may not require special transport system but the free diffusion rate (flip-flop) of the long-chain fatty acids is still questionable (as reviewed in chapter I). The observed inefficient elongation of the long-chain fatty acid could also be interpreted as the long-chain fatty acid export system not being functional in the reverse direction, that is towards the stroma.

More distantly related projects from the plastid envelope proteomics

The work described in chapter IV is not only directed towards the search for lipid transporters but also is aimed for a larger interest in plastid envelope proteins. Plastids draw the attention of plant biologists in large part because of their defining roles in establishing the character of the plant cell. Besides the known important roles of the envelope in various metabolisms, the envelope proteins may hold one key to the

understanding of coordinated control between the plastid and the rest of the cell. The plant research community is moving toward the goal to assign function to each gene products and in order to facilitate the accomplishment of this goal, global analyses of whole genomes are encouraged. Bioinformatics, genome wide expression profiling, proteomics, and reverse genetics are examples of such approach. Assigning the subcellular localization is one of the important steps towards the understanding of the function of any protein (Somerville and Dangl, 2000). Therefore the work of predicting the plastid envelope proteins has a broader perspective for current and future directions of plant science.

The *At*PEM database can be used as a template to expand into a more comprehensive plastid envelope functional proteome database by incorporating rapidly accumulating information such as experimentally confirmed envelope proteins, proteins identified by mass spectroscopy based proteomics approach, T-DNA insertion mutant phenotypes, etc. Also the database can be used as a model to create similar databases for the agriculturally important crops that are being sequenced such as rice and canola. In the future plastid envelope specific gene chips can be designed to study the global expression of envelope proteins.

The selected candidates in the *At*PEM database can also be used in an alternative approach to complement the 'proteomics' approach to identify novel envelope membrane proteins. The candidates can be analyzed for their targeting to the chloroplasts by *in vitro* reconstitution of import into the chloroplast and their partitioning to the envelope

membranes by fractionation studies. This approach can be especially powerful in identifying highly-hydrophobic and low-abundance proteins. Above all it can be used to circumvent the difficulties of preparing 'pure' plastid envelope membranes from various nonphotosynthetic tissues. For example, it is challenging to isolate plastids from Arabidopsis developing seeds and to prepare sufficient plastid envelope membrane samples for a proteomics project (besides the issue of purity). In a work not described in this thesis, I have selected 89 putative developing seed-plastid envelope proteins among the *At*PEM by selecting those with EST's from the developing seed library. Among them were proteins with EST's exclusively from the developing seeds. Some proteins showed more than 2 fold changes during the seed filling according to the developing seed microarray data (these proteins may have important function in the seed filling). Also some of them had only 1 or 2 total EST's suggesting that they are expressed at very low level thus will be difficult to be identified by standard proteomics approach. Gene specific primers for 66 candidates were designed to amplify the cDNA templates by the reverse transcriptase polymerase chain reactions (RT-PCR). The primers were specially designed to contain necessary sequences for the direct use of the amplified cDNA in *in vitro* transcription/translation reactions and subsequently for the plastid targeting experiments. Although time did not allow these experiments to be completed, the approach is clear-cut for delving deeper into characterization of plastid envelope proteins that are specific to seeds.

Future perspectives on fatty acid transport research in plant

Membranes compartmentalize different biosynthetic pathways and the proteins that transport various metabolites across them are potentially important regulatory sites (Flugge, 2000). This might be true for fatty acid synthesis as well. Long-chain acyl CoA's had inhibitory effects on plastidial glucose-6-phosphate transporter and on the adenylated transporter, reducing carbon flux from either glucose-6-phosphate or acetate into long-chain fatty acids in plastids isolated from oilseed rape embryos and from pea roots (Fox et al., 2000; 2001). These inhibitory effects were reversed by the addition of proteins that bind long-chain acyl CoA's implying a mechanism for the regulation of fatty acid synthesis by the ratio between the acyl-CoA binding proteins and acyl-CoA's. Feedback inhibition of fatty acid synthesis was also observed in tobacco suspension cells and the data were interpreted as acetyl-CoA carboxylase being central in the feedback regulation although the exact mechanism is still obscure (Shintani and Ohrogge, 1995). BSA had a positive effect on the overall acetate incorporation rate into the fatty acids by isolated chloroplasts (data not shown). This stimulatory effect of BSA was also observed by Fox et al. (2000) but they explained it through BSA acting as an acyl-CoA binding protein to remove the inhibitory effect of long-chain acyl CoA's on the adenylate transporter (Fox et al., 2000). However the stimulatory effect of BSA was observed even in the absence of coenzyme A or/and ATP (unpublished data). Perhaps the BSA acted positively to the overall acetate incorporation rate by sequestering unesterified fatty acids which posed inhibitory effects on the FAS. These observations suggest that the fatty acid export system may potentially be an important regulatory point. The regulatory aspect of the fatty acid transport is connected with the possibility of genetic engineering and thus warrents future attentions and further investigations.

Further study about the molecular mechanism of fatty acid transport also calls for increased knowledge about plastid structure in general. Although much is known about the plastid double membrane envelope, the molecular resolution of the fine structure remains unresolved. For example, there appear to be no direct connections between the inner and the outer membrane of the plastid envelope although some "contact points" have been observed (Douce and Joyard, 1990). These contact points are mainly thought to be involved in peptide import from the cytosol but it is not known whether these kind of structures are also responsible for the transfer of fatty acid or other molecules between the two membranes. A number of proteins that form the plastid division machineries are arranged concertedly in both the inner and the outer membrane envelope during plastid division. The complexity of this machinery which is beginning to be understood (reviewed in Osteryoung and Nunnari, 2003), in a way predicts other undiscovered mechanistic organizations of the envelope. Another example of such specialized structures on the envelope is the cytoskeletal framework that allows the photorelocation movement of the plastids. Plastids are not static but able to migrate or be arranged into specific orientations in response to the environmental changes, namely, the availability of lights of different wavelengths (reviewed in Wada et al., 2003). The plastid envelope seems to be more dynamic than it has been considered to be in the past. Green fluorescent protein (GFP) imaging techniques have revealed a striking image of long thin tube like structures (called stromules) emanating from the plastid surface (Kohler et al., 1997; Kohler and Hanson, 2000). The stromules can stretch into distant areas in the cells sometimes interconnecting plastids. The stroma localized proteins were observed to flow

through the stromules. Although the function of the stromules is not known they may be involved in intracellular coordination. The increased surface area of the plastid envelope caused by the stromule will affect the free fatty acid diffusion kinetics in those tissues where stromules are observed. Finally, specified zones in the ER that interact with other organelles such as mitochondria, Golgi and plasma membrane were implicated in the interorganellor exchange of lipid molecules in animals and yeasts (Voelker et al., 2003). It is possible that such structure may exist between the plastid and the ER that mediates exchange of certain lipids. These yet to be discovered aspects of plastid biology are essential for complete understanding of fatty acid export from the plastid and for the study of the lipid trafficking in general.

In conclusion, after very little research for decades, lipid transport in plants is now beginning to be studied. This challenging task may uncover many hidden aspects of the lipid biochemistry in plants. The continued accumulation of vast shared information among the science research community will accelerate our search for the lipid transporters in plants. As has occurred so far in other organisms, combining genetic (mutant) analyses with the proper biochemical analyses will continue to make major breakthroughs in this area.

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