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dissertation entitled

HOW PLANTS MANAGE THEIR FATTY ASSETS: A STUDY INTO THE ORGANIZATION AND REGULATION OF THE PLANT FATTY ACID BIOSYNTHETIC PATHWAY

presented by

David Ken Shintani

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in **Botany**

John B. Ohlrogge
Major professor

Date 4/6/96

MSU is an Affirmative Action/Equal Opportunity Institution

O-12771

HOW PLANTS MANAGE THEIR FATTY ASSETS: A STUDY INTO THE ORGANIZATION AND REGULATION OF THE PLANT FATTY ACID BIOSYNTHETIC PATHWAY

By

David Ken Shintani

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1996

ABSTRACT

HOW PLANTS MANAGE THEIR FATTY ASSETS: A STUDY INTO THE ORGANIZATION AND REGULATION OT THE PLANT FATTY ACID BIOSYNTHETIC PATHWAY.

By

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This dissertation represents the cumulative results from several individual projects that pertained to different aspects of the organization and regulation of plant fatty acid biosynthesis. The role of multiple isoforms of acyl carrier protein (ACP) in plant fatty acid synthesis was addressed in studies where the *Arabidopsis* ACPs were characterized. Through these studies, the predominant leaf ACP isoform (LMI ACP) was purified and was used to identify the corresponding cDNA clone. The tissue specific pattern of LMI ACP expression was determined and the possible role of specific ACP isoforms in directing mature fatty acids towards different metabolic fates was discussed. These studies also lead to identification and characterization of an *Arabidopsis* mitochondrial ACP. Immunolocalization and protein import studies were used to confirm the identity the mitochondrial ACP. Furthermore, *in vitro* fatty acid synthase assays indicated that the mitochondrial ACP was able to function as a co-factor for fatty acid synthesis and that this ACP isoform appeared to facilitate the synthesis of medium chain fatty acids. Studies were also conducted to look at the effect of exogenously applied lipid on tobacco cell fatty acid synthesis. These studies revealed that plant fatty acid synthesis is regulated by a product feedback mechanism. Analysis of acyl-ACP intermediates of fatty acid synthesis implied that acetyl-CoA carboxylase (ACCase) was an important regulatory enzyme involved in the feedback inhibition of plant fatty acid synthesis. Additionally,

it was determined that the feedback inhibition was most likely occurring through a biochemical mechanism. Because these and other *in vivo* findings suggested that the plastid ACCase was a key enzyme involved in controlling the flux through fatty acid biosynthesis, experiments were performed to alter the level of ACCase in tobacco. The expression of the tobacco biotin carboxylase subunit of the plastid ACCase was altered using over- and antisense-expression approaches in transgenic plants. The effect of altered biotin carboxylase on the fatty acid synthesis pathway flux and on ACCase complex formation was addressed in these studies. The combined results presented in this dissertation have advanced our understanding of plant fatty acid metabolism and opened new avenues of research in the plant lipid field.

To my parents, Kazumi and Doris Shintani,
whose sacrifices allowed me the luxury
of pursuing a career in science.

ACKNOWLEDGMENTS

Without the support of my best-friend and wife, Christie Howard, I would not have finished my PhD. It is through her strength and encouragement that I was able to make it though the many low points of graduate school and fully enjoy the high points. Our many late night scientific discussion before going to sleep contributed significantly to the work presented in this dissertation. I want to thank Christie for putting up with my poor moods and for picking up the slack at home when I wasn't able to (or was to lazy to) help out. I would also like to thank Christie for reminding me that there is life outside of the laboratory and for encouraging me to pursue that life.

I would like to thank John Ohlrogge for his willingness to take a chance on me and accepting me into his laboratory. I have grown both intellectually and personally through John's guidance. He has been the ideal mentor who, though his contagious enthusiasm, encouraged me to work hard and excel. I am grateful to John for allowing me to pursue my own "hair-brained" ideas and his willingness to discuss issues that went against the grain of the current dogma. I would also like to thank Carol Ohlrogge for her kindness and generosity in inviting Chrisitie and me into her home on several occasions. We have considered Carol and John as part of our surrogate family since we have been in Michigan and are grateful for their support.

My time in the Ohlrogge lab has been very rewarding and this is primarily due to the many friendships that I have established with the past and present

members of this lab. I would like to thank Edgar Cahoon for teaching me how to be a graduate student and for his generosity with his expertise in lipid chemistry. Keith Roessler has been a good friend and the one person that I love to argue with about anything. I also have enjoyed working with Xiaoming Bao, who is also a good friend and good source of cigarettes. I would like to thank Linda Savage for listening to me bitch about everything and nothing at all, Nicki Engeseth for her sympathetic ear, Kathy Schmid for encouraging me to do things that I didn't feel like doing (like writing or preparing for talks), Sergei Mekhedov for his bad Russian jokes, Basil Shorrosh for many intellectual discussions, Sarah Hunter for fixing the computers after I screwed them up, Dusty Post-Beittenmiller for her teaching me to think on my feet, Alenka Housek-Radojcic for her wonderful cakes, Peter Dormann for his biochemical expertise, Paul Roesler for his promising to hire me to work in his hardware store, Ray Pacovsky for his incredible energy, Hajime Wada for continuing the mtACP work, Vicki Eccleston for her good mood, and everybody else (i.e. Kuni Nakahira, Adrian Butt, Oscar de Ilarduya, Becky Peck, James and Amy, Grattan Roughan, Abi, Arif, and anyone I may have forgotten). I would also like to thank Steve Schwartz, Susan Fujimoto, Beth Rosen, Todd and Laura Black, John Shanklin and Jim Dombrowski for their friendship during our stay at MSU. I would also like to thank Jan Jaworski for several helpful discussions during his many visits to East Lansing.

I would also like to thank Pam Green for her support as a committee member and for her generosity in allowing me to use her facilities and equipment. I would also like to thank Natasha Raikhel, Ray Hammerschmidt, and Lee Macintosh for their guidance as members on my graduate committee.

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Introduction

The role of fatty acids in cellular metabolism

Fatty acids are essential compounds needed by almost all biological systems and are involved in several cellular processes. The majority of the fatty acid present in the cell is associated with membrane lipids. Fatty acids make up the hydrophobic component of membrane lipids, whose physical properties, such as melting temperature and packing volume, are greatly influenced by the chain length and the degree of desaturation of the attached fatty acid groups.

Although fatty acids are primarily recognized as structural components of membrane lipids, they are also important in many other cellular processes. Fatty acids are precursors for the synthesis of storage compounds such as fats, oils and waxes (Ohlrogge, 1994; Battey et al., 1989). In plants, fatty acid precursors are necessary for the production of epidermal waxes and cutin, substances that help prevent desiccation of plant tissues and protect against pathogen attack (Kolattukudy, 1987).

Fatty acids and their derivatives have also been implicated as signal molecules involved in several signal transduction pathways. In animal systems, fatty acids precursors are needed for the synthesis of prostaglandins, compounds which have been implicated in a range of biological activities including the perception of pain, tissue inflammation, and smooth muscle contraction (Smith and Borgeat, 1985). Jasmonic acid, a plant signaling molecule associated with the induction of plant defense mechanisms, is synthesized from the fatty acid linolenic acid (18:3) (Vick and Zimmerman, 1984).

Fatty acids are also involved in the post-translational modification of

specific proteins. Protein acylation has been shown to be involved in the targeting and anchoring of proteins to specific membranes (Towler et al., 1988; McIlhinney, 1990; Milligan et al., 1995). This process may also play a role in facilitating the association of different proteins through the interaction of the fatty acid chain of the acylated protein and a hydrophobic domain of a second protein (Towler et al., 1988; McIlhinney, 1990).

Biochemical Reactions of Fatty Acid Synthesis

The biochemical reactions leading to the production of fatty acids have been well studied and are known to occur by the same basic mechanism in all organisms. The elongation of a growing fatty acid chain proceeds through the step-wise addition of 2 carbons to a growing acyl chain. The source of the 2 carbon donor for the elongation of the growing fatty chain is derived from acetyl-CoA. Acetyl-CoA is committed to fatty acid synthesis in the reaction catalyzed by acetyl-CoA carboxylase (ACCase) which facilitates the ATP dependent carboxylation of acetyl-CoA to yield malonyl-CoA. In a reaction catalyzed by malonyl-CoA:ACP transacylase (MCT) the malonyl-group from malonyl-CoA is then transferred to the protein co-factor acyl carrier protein (ACP). ACP provides the scaffolding from which the fatty acid molecule is built and acts as a vehicle to transport the immature fatty acid chain between sequential enzymatic steps in this biosynthetic pathway.

The elongation of the growing fatty acid chain involves the condensation of an acyl-thioester primer with malonyl-ACP. This reaction is catalyzed by a class of enzymes called β-ketoacyl-ACP synthases (KAS). The acyl-thioester primer is transferred to a cysteine residue in the KAS active site which then interacts with the 2 carbon donor malonyl-ACP. Although most of the

condensation reactions use saturated acyl-ACPs as primers for elongation, acetyl-CoA serves as the primer in the first condensation reaction. In yeast and animal FAS, the transfer of the acyl-thioester primer to KAS is catalyzed by the enzyme acetyl-transacylase (ATA). KAS catalyzes the condensation reaction which results in the release of CO_2 and the formation of a β-ketoacyl-ACP species that is 2 carbons longer than the reactant primer.

The β-ketoacyl-ACP intermediate is reduced in a NADPH requiring reaction catalyzed by β-ketoacyl-ACP reductase (KR) to form β-hydroxybutyrl-ACP. Through the loss of H_2O, the hydroxyacyl-ACP intermediate is converted to an enoyl-ACP by β-hydroxyacyl-ACP dehydratase. The enzyme, enoyl-ACP reductase, then catalyzes the NADPH dependent reduction of the enoyl-ACP intermediate which results in a saturated butyryl-ACP species. The plant-enoyl-ACP reductase (ER) is able to use either NADPH or NADH in this reaction.

Depending on the organism, the FAS complex can catalyze seven or eight rounds of elongation resulting in the formation 16 or 18 carbon acyl-ACPs. The final reactions of fatty acid synthesis involve the removal of the mature fatty acid chains from the ACP co-factor. Two different types of reactions are involved in the termination of fatty acid synthesis. First, the acyl-ACP could be cleaved by a acyl-ACP thioesterases to yield a free fatty acid molecule. Alternatively, the acyl group could be transferred from the ACP to an acceptor molecule (such as free CoA yielding an acyl-CoA molecule, or glycerol-3-phosphate (G-3-P)) to form lyso-phosphatidic acid (the first intermediate of glycerolipid synthesis). Animals terminate fatty acid synthesis via a thioesterase, while the termination reaction in fungal fatty acid synthesis involves an acyl-transferase that transfers the mature fatty acid to CoA. In bacteria, the mature fatty acid is transferred from ACP to either the sn-1 or sn-2 position of glycerol. Plants terminate fatty

acid synthesis in reactions catalyzed by acyl-ACP thioesterases and acyltransferases similar to those occurring in bacteria.

Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase) has been identified as an important regulatory enzyme in bacterial (Magnuson et al., 1993), plant (Post-Beittenmiller et al., 1991 & 1992; Page et al., 1994; Shintani & Ohlrogge, 1995) yeast (Kamiryo and Numa, 1973) and animal (Vagelos, 1971) fatty acid synthesis. The reaction catalyzed by ACCase commits carbon in the form of acetyl-CoA towards fatty acid synthesis. ACCase is composed of three subunits: a biotin carboxylase, a biotin carboxyl carrier protein (BCCP), and a transcarboxylase. The formation of malonyl-CoA catalyzed by this enzyme occurs through a two step mechanism. First, the biotin carboxylase subunit catalyzes the activation of HCO_3- by ATP which is then used to carboxylate a biotin prosthetic group of the BCCP subunit. The transcarboxylase subunit then catalyzes the transfer of the carboxyl group from the BCCP subunit to an acetyl-CoA molecule.

The animal, fungal, and bacterial ACCases are localized in the cytosol. Plant ACCases are localized in both the plastid and the cytosol (Alban et al., 1994; Sasaki et al., 1995). The malonyl-CoA produced by the plastid ACCase is utilized primarily for *de novo* fatty acid synthesis, while the plant cytosolic ACCase is utilized in the synthesis of phenylpropanoids (Ebel and Hahlbrook, 1977) and very long chain fatty acid synthesis (Ohlrogge et al., 1978). Plant cytosolic derived malonyl-CoA has also been shown to be needed for the inactivation of 1-aminocyclopropane 1-carboxylate (ACC) (Kionka and Amrhein, 1984).

The organization of the ACCase subunits differs between different

organisms. In animals (Lopez-Casillas et al., 1988), yeast (Al-Feel et al., 1992), and diatoms (Livne and Sukenik, 1990; Roessler, 1990) ACCases are organized as homomeric complexes of single multifunctional polypeptides. The bacterial ACCase is organized in a heteromeric complex composed of four soluble polypeptides: the BCCP subunit, the biotin carboxylase subunit, and two transcarboxylase subunits (Magnuson et al., 1993 and references within). The organization of the plant ACCase has been an issue of great debate for several years. Recent studies have shown that dicots and non-gramineaceous monocots contain a plastid heteromeric ACCase and a cytosolic homomeric ACCase (Alban et al., 1994; Shorrosh et al., 1995; Choi et al., 1995; Sasaki et al., 1995). Gramineaceous monocots, on the other hand, contain homomeric ACCases in both their plastids and cytosol (Egli et al, 1995).

Fatty acid Synthase

I. Organization of the Fatty Acid Synthase Complex

The organization of the individual enzymatic activities that collectively make up the fatty acid synthase (FAS) complex differs significantly among different organisms (Bloch and Vance, 1977). The yeast and animal FAS enzymes are organized as type I enzyme complexes where the individual enzymatic activities are collectively organized on multifunctional polypeptides. The plant and bacteria FAS enzymes are organized as type II complexes where each enzymatic activity is associated with an individual soluble enzyme.

Type I FAS Complexes

The animal and fungal FAS enzymatic activities are organized on large mutifunctional polypeptides and are referred to as type I enzyme complexes. The

animal FAS complex is organized as a homodimer with a native molecular mass of greater than 500,000 (Wakil, 1989). Each complex is composed of two identical subunits with molecular weights of 260,000. Results from limited proteolysis studies, showed that each animal FAS subunit was composed of three major domains; designated as FAS domains I, II, and III; with respective molecular weights of 127,000, 107,000, and 33,000 (Mattick et al., 1983a). The largest domain, domain I, contained the following enzymatic activities: β-ketoacyl synthase (KAS); malonyl transacylase (MCT); and acetyl transferase (ATA) (Mattick et al., 1983a). Domain II contained the ACP protein co-factor, and the β-ketoacyl reductase, β hydroxyacyl dehydratase, and enoyl reductase activities (Mattick et al., 1983a). The 33 kD domain III, contained the thioesterase activity (Mattick et al., 1983b).

The fungal FAS complex is composed of two different multifunctional subunits, the 212,000 D α-subunit and the 203,000 D β-subunit (Stoops et al, 1978). The α-subunit contains the KAS, β-ketoacyl reductase activities and the ACP moiety (Stoops and Wakil, 1978). The β-subunit contains the β-hydroxyacyl dehydratase, enoyl reductase, MCT, ATA, and acyl-transferase activities (Stoops and Wakil, 1978). The yeast native FAS complex has a $\alpha_6\beta_6$ configuration with a molecular weight of 2,400,000 (Stoops and Wakil, 1980).

The animal and fungal FAS complexes are capable of only 7 rounds of elongation to produce the 16 carbon saturated acyl-ACP, palmitoyl-ACP. The terminal reaction of the animal FAS involves the hydrolysis of the acyl-ACP thioester linkage resulting in free palmitate. This fatty acid is rapidly converted to an acyl-CoA through the action of acyl-CoA synthetase. The terminal reaction of the fungal FAS involves an acyl-ACP transacylase which catalyzes the transfer of the palmitoyl-group from ACP to CoA. In both animal and fungal

cells, the palmitoyl-CoA can then be directed towards glycerolipid synthesis or be further elongated to stearoyl-CoA by a fatty acyl-CoA elongase that is localized on the cytosolic face of the endoplasmic reticulum. The stearoyl-CoA can then serve as a substrate for glycerolipid synthesis or undergo desaturation by stearoyl-CoA desaturase to yield oleyl-CoA.

Type II FAS Complexes

The plant and bacterial FAS complexes are comprised of individual soluble enzymes and are referred to as type II enzyme complexes (Vagelos, 1974; Shimakata and Stumpf, 1982a). Although type II FAS complexes may appear to be less sophisticated then the animal or yeast type I FAS complexes, their organization is certainly more complex. A functional type II FAS complex requires the association of at least nine individual soluble enzyme activities. These enzymes must be coordinated to catalyze the correct reaction in the correct sequence. Additionally, plants and bacteria possess multiple isoforms of certain FAS components, some of which are responsible for specialized functions.

It is not known how the individual FAS subunits are associated with one another in a type II complex. Isolation of an intact FAS complex from bacterial or plant cell extracts has proven difficult because FAS components are so easily disassociated (Shimakata and Stumpf, 1982b). It is possible that the soluble enzymes comprising the type II FAS complex are arranged as loosely associated ordered complex. The components of other multienzyme pathways, such as the citric acid cycle (Srere et al., 1987) and the Calvin cycle (Gontero et al., 1988; Suss et al., 1993), have been reported to be organized in a sequential fashion to form functional complexes. Although little evidence has been obtained to support

the hypothesis that the type II FAS enzymes are organized in an ordered complex, results from *in vitro* plant FAS assays suggest that FAS components in cell free extracts need to reassociate in order to function. It has been shown that the addition of exogenous ACP is necessary to obtain high rates of FAS from cell free extracts. Rutkoski and Jaworski (1978) observed that when exogenous ACP was added to a spinach chloroplast extract, a 15 minute lag period was observed before ^{14}C-malonyl-CoA incorporated into fatty acids. This lag period was eliminated if the extract was pre-incubated with the exogenous ACP for 15 minutes prior to the addition of label, suggesting that the lag period was necessary for FAS complex formation.

Like the animal and yeast type I FAS, the bacterial and plant type II FAS are able to synthesize palmitic acid. However, because type II FAS complexes are much more fluid than the type I FAS, additional enzymes with specialized functions are able to integrate into the type II complex and allow for the production of a variety of different fatty acid products. For example, plants possess a specific KAS isoform, KAS II that is responsible for the elongation of palmitoyl-ACP (16:0-ACP) to stearoyl-ACP (18:0-ACP) (Shimakata and Stumpf, 1982c). The plant 18:0-ACP can then be modified by a soluble acyl-ACP desaturase that can introduce a single double bond at the Δ9 position to produce oleoyl-ACP (18:1Δ9-ACP) (McKeon and Stumpf, 1982). Each of these long chain acyl-ACPs can be cleaved by a thioesterase to produce palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1Δ9) products (McKeon and Stumpf, 1982). These products are then esterified to CoA and subsequently serve as substrates for glycerolipid biosynthesis (Roughan and Slack, 1977). *E. coli* cells contain two KAS isoforms, KAS I and II, that are involved in the synthesis of long chain saturated and mono-unsaturated fatty acids (Garwin et

al., 1980). Both *E. coli* KAS isoforms can catalyze the condensation reactions leading to the synthesis of 16:0-ACP, however, KAS I is also capable of elongating the mono-unsaturated 10 carbon fatty acyl-ACP, cis-3-decenoyl-ACP, in a series of condensation reactions to produce the mono-unsaturated 16 carbon fatty acyl-ACP, palmitoleoyl-ACP (16:1Δ9-ACP). 16:1Δ9-ACP can then be elongated by KAS II to form cis-vaccenoyl-ACP (18:1Δ11-ACP). The *E. coli* long chain acyl-ACPs are then rapidly converted to acyl-CoAs by acyltransferases and these acyl-CoAs subsequently enter gylcerolipid synthetic pathways.

The type II configuration also allows plants and bacteria to alter the production of certain fatty acid species in order to respond to environmental changes. *E. coli* is known to alter the fluidity of their membranes in response to fluctuations in temperature by modulating the synthesis of the two mono-unsaturated fatty acids cis-vaccinic acid (18:1Δ11) and palmitoleic acid (16:1Δ9) such that at elevated temperatures, the ratio of 18:1Δ11 to 16:1Δ9 levels decreases, while at low temperatures this ratio increases (Garwin and Cronan, 1980). This change in *E. coli* membrane lipid fatty acid composition was attributed to the thermoregulation of KAS II, the enzyme responsible for the elongation of 16:1Δ9-ACP to 18:1Δ11-ACP. It was shown that the increased KAS II activity at lower temperatures was due to the enzymes unusually low temperature optimum and not due to changes in the levels of KAS II mRNA or protein. Plant cell suspension cultures have also been reported to alter the synthesis of 18 carbon acid species in response to changes in temperature and the addition of exogenous fatty acids (MacCarthy and Stumpf, 1980a). In plants, 18 carbon fatty acids make up the majority of the unsaturated fatty acid species while the saturated fatty acid, 16:0, is the most abundant 16 carbon fatty acid

species. It observed that plant suspension cultures altered the fluidity of their membranes in response to increased temperature by decreasing the degree of membrane lipid fatty acid desaturation. It was shown that KAS II was labile at elevated temperatures which let to an increase in the ratio of 16 carbon to 18 carbon fatty acids (MacCarthy and Stumpf, 1980b).

The type II FAS configuration also allows for the introduction of FAS enzymes with specialized functions into the normal FAS complex to produce unusual fatty acid products. One such example can be seen in plants that produce oils containing high levels of medium chain fatty acids. The production of lauric acid (12:0) in the fruits of the California Bay Laurel tree has been attributed to an unusual acyl-ACP thioesterase that specifically cleaves fatty acids from acyl-ACPs of 12 carbons in length. cDNA clones corresponding to the lauryl-ACP thioesterase have been expressed in both *E. coli* (Ohlrogge et al., 1995) and *Brassica napus* (Voelker et al., 1992), two species which do not normally produce medium chain fatty acids. In both cases, it appeared that the medium chain acyl-ACP thioesterase was able to integrate into the host FAS complex and produce medium chain fatty acids. Additionally, Cahoon et al. (1992), were able to express a coriander cDNA clone corresponding to a Δ4-palmitoyl-ACP desaturase which was involved in the synthesis of an unusual fatty acid known as petroselinic acid in tobacco cells and observe the synthesis of this unusual fatty acid product.

II. Subcellular Localization of the Fatty Acid Synthase Complex

While the animal, fungal and bacterial FAS activities are predominantly localized with in their respective cytosols, the plant FAS is predominantly associated with the plastid compartment (Ohlrogge, 1979). The plastid

localization of a prokaryotic type FAS I complex in plant cells is thought to have originated through the endosymbiotic relationship between an eukaryotic single cell organism and a photosynthetic bacteria. For this reason, *Anabaena*, a cyanobacterium which is known to contain a type II FAS, has been used by some researchers as a model system to study plant fatty acid synthesis. *Euglena*, a single cell green algae that contains both a cytoplasmic type I FAS and a type II plastid localized FAS (Hendren and Bloch, 1979), may represent an organism that is closely related to the hypothetical photosynthetic endosymbiotic organism.

Evidence has been emerging for the presence of a type II FAS in the mitochondria of animals, fungi, and plants. The work of Brody and Mikolajczk (1988) showed that *Neurospora crassa* mitochondria contained a soluble low molecular weight ACP. Antibodies made to the *Neurospora* ACP, were reported to cross- react with protein extracts isolated from plant mitochondria (Chuman and Brody, 1989). Mitochondrial ACPs have been purified to homogeneity from *Neurospora* (Sackmann et al., 1991) and bovine heart muscle (Runswick et al., 1991) mitochondria and cDNA clones corresponding to these (Runswick et al., 1991; Sackmann et al., 1991) and the *Arabidopsis* (Shintani and Ohlrogge, 1994) mitochondrial ACP have recently been cloned. Radiolabeling experiments done with isolated *Neurospora* mitochondria suggest that these mitochondria may be capable of *de novo* fatty acid synthesis (Mikolajczyk and Brody, 1990; Zenson et al., 1992). In further support of a mitochondrial fatty acid synthase, Harrington et al. (1993), reported the identifitcation of a β-ketoacyl synthase that is localized within yeast mitochondria.

Why a mitochondrial localized FAS exists is unclear since it has been shown that cytosolic FAS, in the cases of yeast and animals, and the plastid

FAS, in the case of plants, are sufficient to meet the cellular demand for fatty acid synthesis. It has been proposed that a mitochondrial FAS may be involved in the synthesis of mitochondrial specific lipids such as cardiolipin (Brody et al., 1990). It is also possible that the mitochondrial ACP may be involved in cellular processes other than fatty acid synthesis. Recently, researchers studying the mitochondrial electron transport have reported that the mitochondrial ACP may be a component of the NADH:ubiquinone oxidoredutase (complex I) (Runswick et al., 1991; Sackmann et al., 1991). It has been shown in that the disruption of the gene coding for the mitochondrial ACP lead to the inability of the NADHP:ubiquinone oxidoreductase (complex I) to form (Schulte et al., 1994) suggesting that the mitochondrial ACP may be a structural component of complex I of the mitochondrial electron transport chain.

Regulation of Plant Fatty Acid Synthesis

Although the biochemistry of plant fatty acid synthesis has been thoroughly studied, relatively little is known about the regulation of this pathway. Plants appear to synthesize only enough fatty acids to satisfy the demand of glycerolipid synthetic pathways. This is clearly exemplified by the observation that the accumulation of unesterified fatty acids is never observed in plants. The high degree of FAS regulation acts to prevent the over production of fatty acids which would be energetically wasteful and could lead to an accumulation of fatty acids or acyl-CoAs whose detergent nature would be detrimental to the cell. It appears that high rates of fatty acid synthesis are primarily observed in tissues where high rates of glycerolipid synthesis are occurring, such as developing oilseeds and rapidly dividing and expanding cells. Although the rates of fatty acid synthesis are significantly lower in mature

differentiated cells, these rates appear to be sufficient for the maintenance and repair membrane lipids.

Modes of fatty acid synthesis regulation in plants.

Plants fatty acid synthesis is regulated through both biochemical and genetic mechanisms. The biochemical regulation of fatty acid synthesis allow plants to respond immediately to changes in the demand for fatty acids caused by short term fluctuations in environmental conditions or metabolite levels. The genetic regulation of fatty acid synthesis allows the plant to respond to long term changes in cellular demand for fatty acids by increasing the amount of the biosynthetic machinery. Depending on the specific species or tissue, one or a combination of the two types of regulation may be involved in controlling fatty acid synthesis.

Biochemical Regulation of Fatty Acid Synthesis

The rates of fatty acid synthesis in plants are strongly influenced by factors effecting both the metabolic state of the cell and the fluidity of cellular membranes. Fatty acid synthesis is an energetically costly process, such that the synthesis of one mole of palmitic acid (16:0) requires 8 moles of acetyl-CoA, 7 moles of ATP, 7 moles of NADH, and 7 moles of NADPH.

Biochemical regulation typically occurs through covalent or allosteric modifications which either enhance or attenuate the activity of specific regulatory enzymes. These modifications effecting enzymatic activity are usually fully reversible. In contrast to the regulation of synthetic pathway by the induction of gene expression, which occurs within the range of tens of minutes, biochemical modes of regulation occur much more rapidly, within the range of

fractions of minutes.

Much effort has been expended to identify enzymes that regulate the flux of carbon through fatty acid synthesis. Researchers have tried to identify the rate limiting enzymes in fatty acid synthesis by comparing the *in vitro* activities of FAS enzymes measured from plant extracts. Of the FAS enzymes surveyed, the *in vitro* velocity of the reaction catalyzed by acetyl-CoA:ACP transacylase (ATA), was by far the slowest and was therefore putatively identified as the rate limiting enzyme controlling fatty acid biosynthesis in plants (Shimakata and Stumpf, 1983). However, later studies suggested that ATA may play only a minor role in plant fatty acid synthesis and may actually catalyze a side reaction of the condensing enzyme KAS III (Jaworski et al., 1993). These findings indicate that although *in vitro* measurements of enzymatic rates can sometimes help identify regulatory enzymes within a pathway, in many cases the conclusions based on these measurements may not apply to what is occurring *in vivo*.

Recently, *in vivo* methods have been used to monitor changes in the levels of FAS intermediates that occurred in response to changes in the flux of carbon through the fatty acid synthetic pathway. These methods take advantage of the fact that all FAS intermediates are covalently liked to the protein co-factor ACP. Because acyl-ACP intermediates are extremely labile, with half lives in the range of seconds, purification protocols had to be developed that would ensure the stability of these intermediates (Post-Beittenmiller et al., 1991). The purified acyl-ACP intermediates could be fractionated on non-denaturing polyacrylamide gels containing varying concentrations of urea. The relative mobility of individual acyl-ACP intermediates in this gel system was shown to be highly dependent on the nature of the attached acyl-group. The identity of these acyl-

ACP intermediates could then be determined by their co-migration with *in vitro* synthesized acyl-ACP standards. By analyzing the accumulation or depletion of specific acyl-ACP intermediate pools, it is possible to identify specific enzymatic steps which were involved in the regulation of this pathway.

I. Light / Dark Regulation of Plant Fatty Acid Synthesis

One example of the biochemical regulation of fatty acid biosynthesis is the light/dark regulation of fatty acid synthesis in leaves. It has been shown that the rate of fatty acid synthesis is induced 5-7 fold when plants are transferred from the dark into the light (Browse et al., 1981). The light activation of FAS occurs within seconds after the transfer to the light and is rapidly reversed when plants are transferred back to the dark.

When acyl-ACP and acyl-CoA pools were analyzed from light and dark treated spinach leaves and chloroplasts, acetyl-CoA carboxylase (ACCase) was identified as an important enzyme involved in the dark/light regulation of plant FAS (Post-Beittenmiller et al., 1991 and 1992). These studies showed that the levels of malonyl-CoA (the product of ACCase) and malonyl-ACP (which is thought to be in equilibrium with malonyl-CoA) increased when leaves or chloroplast that were incubated in the light (Post-Beittenmiller et al., 1991; Post-Beittenmiller et al., 1992), indicating that ACCase activity was stimulated by the light treatment. It has also been shown that ACCase activity is significantly higher in extracts isolated from When ACCase activity was measured rapidly

Although the mechanism by which ACCase is regulated by light is not well understood, Nakahira and Ohlrogge (manuscript in prep) showed that the chloroplast electron transport system was somehow involved in the light activation of ACCase in isolated spinach chloroplast. Using various

combinations of artificial electron donors and acceptors they showed that electron flow between the cytochrome b_6/f-plastocyanin region and photosystem I was required for the light activation of ACCase. Furthermore, they showed that a membrane potential, but not a pH gradient across the thylakoid membrane was required for the light activation of ACCase. In experiments where the thylakoid membrane potential was dissipated by the addition of the specific K^+ ionophore valinomycin, the light activation of ACCase was not observed. However, when the pH gradient across the thylakoid membrane was disrupted by the addition of NH_4Cl, the light activation of ACCase was not effected. The relationship between electron transport, the establishment of a membrane potential, and the light activation of ACCase are not well understood.

Interestingly, the electron transport to photosystem I has also been shown to be required for the activation of ribulose bisphosphate carboxylase (RUBISCO) by RUBISCO activase (Campbell and Ogren, 1990). However, in contrast to the light activation of ACCase, Campbell and Ogren (1990) showed that the activation of RUBISCO required a pH gradient but does not require the establishment of a thylakoid electron potential. It is possible that the light activation of ACCase and RUBISCO may be occurring through similar mechanism. However, as of yet, how electron transfer to photosystem I is involved in the activation of either ACCase or RUBISCO is unknown.

II. Product Feedback Inhibition of Plant Fatty Acid Synthesis

A second example of biochemical regulation of plant fatty acid synthesis can be seen during product feedback inhibition. It has been shown that the addition of exogenous fatty acids in the form of Tween-fatty acid esters can cause a significant decrease in the rates of fatty acid synthesis in soybean and

tobacco suspension cultures (Terzaghi et al., 1985; Terzaghi, 1986; Shintani and Ohlrogge, 1995). Rates of fatty acid synthesis decreased by 3 - 5 fold within 15 minutes of the addition of oleoyl-Tween (Shintani and Ohlrogge, 1995). By analyzing the pools of acyl-ACPs in Tween treated and control cells, it was shown that ACCase played an important role in regulating fatty acid synthesis during product feedback inhibition (Shintani and Ohlrogge, 1995). Due to rapid onset of the inhibition and the observation that protein and mRNA levels of the subunits of the plastid ACCase and FAS enzymes did not change during the feedback inhibition, it appeared that the regulation of ACCase during product feedback inhibition was probably occurring through a biochemical mechanism rather than a genetic mechanism.

The identity of the feedback molecule was not determined, nor was the mechanism by which ACCase was inhibited. What was clear from the analysis of the acyl-ACP pools was that long chain acyl-ACPs were probably not acting as the feedback molecules effecting ACCase. This assumption was based on the observation that upon the onset of the feedback inhibition, the pools of long chain acyl-ACPs become rapidly depleted. One might predict that if the long chain acyl-ACPs were acting as feedback molecules, that the pools of these acyl-ACPs would increase instead of decreasing as was observed. Furthermore, no inhibition was detected in studies where long chain acyl-ACPs were added to partially purified plastid ACCase (Roesler et al., 1995).

III. Role of ACCase in Controlling the Flux Through Fatty Acid Synthesis in Maize Leaves.

Specific enzyme inhibitors have also been used to study potential regulatory enzymatic steps in fatty acid synthesis. Page et al. (1994) studied the

effect of two highly specific inhibitors of the maize plastid ACCase, sethoxydim and fluazifop, on rates of fatty acid synthesis. By correlating changes in rates of ACCase activity due to the addition of either of the two inhibitors with changes in the rate of fatty acid synthesis, they were able to show that ACCase exerted a high degree of control fatty acid biosynthesis.

Molecular Approaches to Understanding the Biochemical Regulation of Fatty acid Synthesis

Recently, many of the genes coding for components of the plant fatty acid biosynthetic machinery have been cloned. This development should allow for rapid advancements leading to a better understanding of the biochemical mechanisms involved in the regulation of this central biosynthetic pathway. Because it is now possible to alter levels of specific enzymes within the fatty acid synthetic pathway in transgenic plants and determine what effect these changes would have on pathway flux, we should now be able to use the principles of metabolic control theory to test our assumptions about possible regulatory points within the plant fatty acid synthetic pathway. The major idea behind metabolic control theory is based on the assumption that the flux through a given synthetic pathway is controlled through the cumulative control of the sum of the pathway enzymes. Therefore, if the level of a particular enzyme is changed, the degree of change in the over-all flux through the pathway caused by this change in enzyme level reflects the degree of control that the enzyme has over the entire pathway. So if a small change in the levels of a particular enzymes causes large changes in the flux through the pathway, this enzyme has large degree of control over the pathway. Alternatively, if it is necessary to make large changes in enzyme levels to make large changes in pathway flux, then the

enzyme will have a low degree of control over flux through the pathway.

Genetic Regulation of Fatty Acid Synthesis in Plants

Several examples of the genetic regulation of fatty acid synthesis has been shown to occur in plants. These include the increased gene expression of FAS related proteins during the period of early leaf development and prior to the period of oil deposition in oilseeds (Hannapel and Ohlrogge, 1988). In each of these cases the change in the rate of fatty acid synthesis has been attributed to an increase in the levels of FAS related enzymes.

Fatty acid synthesis has been reported to occur at significantly higher rates in young, expanding spinach leaves than in older mature spinach leaves (Kannagara et al., 1973). This increased rates of fatty acid synthesis corresponded to increase in the level of FAS machinery, which was evident by an increase in the mRNA and protein levels of ACP (Hannapel and Ohlrogge, 1988). Additionally, the increased rate of fatty acid synthesis observed in developing seeds during the period of oil deposition has been shown to be due to an increase in FAS related gene expression. The levels of ACP mRNA (Hannapel and Ohlrogge, 1988) and protein (Ohlrogge and Kuo, 1984) were shown to increase to high levels just prior to the onset of oil synthesis in developing soybean seeds. Furthermore, in developing *Brassica napus* seed, the profile of enoyl-ACP reductase and stearoyl-ACP desaturase mRNA accumulation coincides with the period of increased FAS activity (Fawcett et al., 1994). These observations suggest that the genetic regulation of FAS during seed development may involve the coordinate increase in the synthesis of the entire FAS machinery.

So far very little is known about cis- or trans-acting factors involved in the

transcriptional induction of genes coding for components of fatty acid synthetic machinery. This may be due to the fact that until recently, few of the genes encoding proteins involved in fatty biosynthesis have been cloned from plants. However, fairly extensive studies have been done to characterize the promoter region of *Arabidopsis* ACP genes (Baerson and Lamppa, 1993; Baerson et al., 1994) Through this work, cis elements have been identified in the promoter region of a constitutively expressed *Arabidopsis* ACP gene that are necessary for leaf, root, and seed expression (Baerson et al., 1994). Although advancements in this area have been slow in coming as of late, the recent cloning of many interesting cDNAs and genes involved in fatty acid synthesis (Topfer and Martini, 1994) should lead to the rapid growth of this particular area.

Literature Cited

Al-Feel W, Chirala SS, Wakil SJ (1992) Cloning of the yeast FAS3 gene and primary structure of yeast acetyl-CoA carboxylase. Proc Natl Acad Sci USA **89**: 4534-4538.

Alban C, Baldet P, Douce R (1994) Localization and characterization of two structurally different forms of acetyl-CoA carboxylase in young pea leaves, of which one is sensitive to aryloxyphenoxypropionate herbicides. Biochem J **300:** 557-565.

Baerson SR, Lamppa GK (1993) Developmental regulation of an acyl carrier protein gene promoter in vegatitive and reproductive tissues. Plant Molec Biol **22**: 255-267.

Baerson SR, Vanderheiden MG, Lamppa GK (1994) Identification of domains in an *Arabidopsis* acyl carrier protein gene promoter required for maximal organ-specific expression. Plant Molec Biol **26:** 1947-1959.

Battey JF, Schmid KM, Ohlrogge JB (1989) Genetic engineering for plant oils: potential and limitations. TIBTECH **17:** 122-126.

Bloch K, Vance D (1977) Control mechanisms in the synthesis of saturated fatty acids. Ann Rev Biochem **46:** 263-298.

Brody S, Mikolajczyk S (1988) *Neurospora* mitochondria contain an acyl carrier protein. Eur J Biochem **173:** 353-359.

Brody S, Mikolajczyk S, Chuman L (1990) Studies on *de novo* fatty acid synthesis in mitochondria. In PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure, and Utilization. Portland Press, Ltd, London, pp 117-119.

Browse J, Roughan PG, Slack CR (1981) Light control of fatty acid synthesis and diurnal fluctuations of fatty acid composition in leaves. Biochem J **196:** 347-354.

Campbell WJ, Ogren WL (1990) Electron transport through photosystem I stimulates light activation of ribulose bisphosphate carboxylase/oxygenase (RUBISCO) by RUBISCO activase. Plant Physiol **94:** 479-484.

Cahoon EB, Shanklin J, Ohlrogge JB (1992) Expression of a coriander desaturase results in petroselininc acid production in transgenic tobacco. Proc Natl Acad Sci USA **89:** 11184-11188.

Choi JK, Yu F, Wurtele ES, Nikolau BJ (1995) Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of the chloroplastic acetyl-CoA carboxylase. Plant Physiol **109:** 619-625.

Chuman L, Brody S (1989) Acyl carrier protein is present in the mitochondria of plants and eukaryotic micro-organisms. Eur J Biochem **184:** 643-649.

Fawcett T, Simon WJ, Swinhoe R, Shanklin J, Nishida I, Christie WW, Slabas AR (1994) Expressionof mRNA and steady-state levels of protein isoforms of enoyl-ACP reductase from *Brassica napus*. Plant Molec Biol **26:** 155-163.

Ebel J, Hahlbrock K (1977) Enzymes of flavone and flavonolgycoside biosynthesis. Coordinateand selective induction in cell-suspension cultures of *Petroselinum hortense*. Eur J Biochem **75:** 201-209.

Egli M, Lutz S, Gengenbach B (1995) cDNA sequence of a multifunctional acetyl-CoA carboxylase from maize. Absract in the proceedings from the 1995 Biochemistry and Molecular Biology of Plant Fatty Acids and Glycerolipids Symposium.

Garwin JL, Klages AL, Cronan JE (1980) Structural, enzymatic, and genetic studies of beta-ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli*. J Biol Chem **255:** 11949-11956.

Garwin JL, Cronan JE (1980) Thermal modulation of fatty acid synthesis in *Escherichia coli* does not involve *de novo* synthesis. J Bacteriol **141:** 1457-1459.

Gontero B, Cardenas ML, Ricard J (1988) A functional five-enzyme complex of chloroplasts involved in the Calvin cycle. Eur J Biochem **173:** 437-443.

Hannapel DJ, Ohlrogge JB (1988) Regulation of acyl carrier protein messenger RNA levels during seed and leaf development. Plant Physiol **86:** 1174-1178.

Harrington A, Hebert CJ, Tung B, Getz GS, Slonimski PP (1993) Identification of a new nuclear gene (*CEM1*) encoding a protein homologous to a beta-keto-acyl synthase which is essential for mitochondrial respiration in *Saccharomyces cerevisiae*. Molecular Microbiology **9:** 545-555.

Hendren RW, Bloch K (1979) Fatty acid synthetases from Euglena gracilis: separation of component activiteis of the ACP-dependent fatty acid synthetase and partial purification of the beta-ketoacyl-ACP reductase. J Biol Chem **255:** 1504-1508.

Jaworski JG, Post-Beittenmiller D, Ohlrogge JB (1993) Acetyl-acylacrrier protein is not a major intermediate in fatty acid biosynthesis in spinach. Eur J Biochem **273:** 981-987.

Kamiryo T, Numa S (1973) Reduction of acetyl-CoA carboxylase content of *Saccharomyces cerevisiae* by exogenous fatty acids. FEBS Letters **38:** 29-32.

Kannangara CG, Jacobson BS, Stumpf PK (1973) Fat metabolism in higher plants. LVII: A comparison of fatty acid-synthesizing enzymes in chloroplast isolated from mature and immature leaves of spinach. Plant Physiol. **52:** 156-161.

Koinka C, Amrhein N (1984) The enzymatic malonylation of 1-aminocyclopropane-1-carboxylic acid in homogenates of mung bean hypocotyls. Planta **162:** 226-235.

Kolattukudy PE (1987) Lipid-derived defensive polymers and waxes and their role in plant-microbe interaction. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A comprehensive treatise, Vol 9, Academic Press, New York, pp 291-314.

Livne A, Sukenik (1990) Acetyl-CoA carboxylase from the marine prymnesiophyte *Isochrysis galbana*. Plant Cell Physiol 31: 851-858.

Lopez-Casillas F, Bai DH, Luo X, Kong IS, Hermodson MA, Kim KH (1988) Structure of the coding sequence and primary amino-acid sequence of acetyl-CoA carboxylase. Proc Natl Acad Sci USA **85:** 5784-5788.

MacCarthy JJ, Stumpf PK (1980) The effect of different temperatures on fatty acid synthesis and polyunsaturation in cell suspension culture. Planta **147:** 389-395.

MacCarthy JJ, Stumpf PK (1980) Incorporation of [2-^{14}C]malonyl-CoA into fatty acids by a cell free extract of *Catharanthus roseus* suspension culture cells. Planta **150:** 412-418.

Magnuson K, Jackowski S, Rock CO, Cronan JE (1993) Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiological Reviews **57:** 522-542.

Mattick JS, Tsukamoto Y, Nickless J, Wakil SJ (1983a) The architecture of the animal fatty acid synthetase: I. Proteolytic dissection and peptide mapping. J Biol Chem **258:** 15291-15299.

Mattick JS, Nickless J, Mizugaki M, Yang CY, Uchiyama S, Wakil SJ (1983b) The architecture of the animal fatty acid synthetase: II. Separation of the core and thioesterase functions and determination of the N-C orientation of the subunit. J Biol Chem **258:** 15300-15304.

McIlhinney RAJ (1990) The fats of life: the importance and function of protein acylation. TIBS **15:** 387-391.

McKeon TA, Stumpf PK (1982) Purification and characterization of the stearoyl-acyl carrier protein desaturase and acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J Biol Chem **257:** 12141-12147.

Mikolajczk S, Brody S (1990) *De novo* fatty acid synthesis mediated by acyl carrier protein in *Neurospora crassa* mitochondria. Eur J Biochem **187:** 431-437.

Milligan G, Parenti M, Magee AI (1995) The dynamic role of palmitylation in signal transduction. TIBS **20:** 181-186.

Nakahira K, Ohlrogge JB (manuscript in preparation) Photosystem I involvement in regulation of acetyl-CoA carboxylase activity in spinach chloroplast.

Ohlrogge JB, Savage L, Jaworski J, Voelker T, Post-Beittenmiller D (1995) Alteration of acyl-acyl carrier protein pools amd acetyl-CoA carboxylase expression in *Escherichia coli* by a plant medium chain acyl-acyl carrier protein thioesterase. Arch Biochem Biophys **317:** 185-190.

Ohlrogge JB (1994) Design of new plant products: Engineering of fatty acid metabolism. Plant Physiol **104:** 821-826.

Ohlrogge JB, Kuo TM (1984) Control of lipid synthesis during soybean seed development: enzymic and immunochemical assay of acyl carrier protein. Plant Physiol **74:** 622-625.

Ohlrogge JB, Kuhn DN, Stumpf PK (1979) Subcellular localization of acyl carrier protein in leaf protoplast of *Spinacia oleracea*. Proc Natl Acad Sci USA **76:** 1194-1198.

Ohlrogge JB, Pollard MR, Stumpf PK (1978) Studies on the biosynthesis of waxes by developing jojoba seed tissue. Lipids **13:** 203-210.

Page RA, Okada S, Harwood JL (1994) Acetyl-CoA carboxylase exerts strong flux control lipid synthesis in plants. Biochim biophys Acta **1210:** 369-372.

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) *In vivo* pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem **266:** 1858-1865.

Post-Beittenmiller D, Roughan PG, Ohlrogge JB (1992) Regulation of plant fatty acid biosynthesis: analysis of acyl-CoA and acyl-ACP substrate pools in spinach and pea chloroplast. Plant Physiol **100:** 923-930.

Roesler KR, Savage L, Shintani DK, Shorrosh BS, Ohlrogge JB (1996) Co-purification, co-immunoprecipitation, and coordinate expression during oilseed development of acetyl-CoA carboxylase activity, biotin carboxylase proetin and biotin carboxyl carrier protein in higher plants. Planta **198:**(in press).

Roessler PG (1990) Purification and characterization of acetyl-CoA carboxylase from the diatom *Cyclotella crypitca*. Plant Physiol **92:** 73-78.

Roughan PG, Slack CR (1977) Long chain acyl-coenzyme A synthestase activity of spinach chloroplast is concentrated in the envelope. Bichem J **158:** 593-601.

Runswick MJ, Fearnley IM, Skehel JM, Walker JE (1991) Presence of an acyl carrier protein in NADH:ubiquinone oxidoreductase from bovine heart mitochondria. FEBS Lett **286:** 121-124.

Rutkoski A, Jaworski JG (1978) Fatty acid synthetase from chloroplast of soybean cotyledons: ACP activation and CoA inhibition. Biochem Biophys Res Commun **84:** 428-430.

Sackmann U, Zensen R, Rohlen D, Jahnke U, Weiss H (1991) The acyl carrier protein in *Neurospora crassa* mitochondria is a subunit of NADH:ubiquinone oxidoreductase (complex I). Eur J Biochem **200:** 463-469.

Sasaki Y, Konishi T, Nagano Y (1995) The compartmentation of acetyl-CoA carboxylase in plants. Plant Physiol **108:** 445-449.

Schulte U, Fecke W, Krull C, Nehls U, Schmiede A, Schneider R, Ohnishi T, Weiss H (1994) In vivo dissection of the mitochondrial respiratory NADH:ubiquinone oxidoreductase (complex I). Biochim et Biophysica Acta **1187:** 121-124.

Shimakata T, Stumpf PK (1983) The purification and function of acetyl-CoA:acyl carrier proetein transferase. J Biol Chem **258:** 3592-3598.

Shimakata T, Stumpf PK (1982b) The prokaryotic nature of the fatty acid synthetase of developing Carthamus tinctorius L. (saffflower) seed. Arch Biochem Biophys **217:** 144-154.

Shimakata T, Stumpf PK (1982b) Fatty acid synthase of *Spinacia oleracea* leaves. Plant Physiol **69:** 1257-1262.

Shimakata T, Stumpf PK (1982c) Isolation and function of spinach leaf beta-ketoacyl-[acyl carrier protein] synthases. Proc Natl Acad Sci USA **79:** 5808-5812.

Shintani DK, Ohlrogge JB (1994) The characterization of a mitochondrial acyl carrier protein isoform isolated from *Arabidopsis thaliana*. Plant Physiol **104:** 1221-1229.

Shintani DK, Ohlroggge JB (1995) Feedback inhibition of fatty acid synthesis in tobacco suspension cells. The Plant Journal **7:** 577-587.

Shorrosh BS, Roesler KR, Shintani D, van de loo FJ, Ohlrogge JB (1995) Structural analysis, plastid localization, and expression of the biotin carboxylase subunit of acetyl-CoA carboxylase from tobacco. Plant Physiol **108:** 805-812.

Smith WL, Borgeat P (1985) The eicosanoids: Prostaglandins, thromboxanes, leukotrienes, and hydroxy-eicosaenoic acids. *In* DE Vance, JE Vance, eds, Biochemistry of Lipids and Membranes, The Benjamin/Cummings Publishing Company, Inc, Menlo Park, CA, pp 325-360.

Srere PA, Sumegi B, Sherry D (1987) Organizational aspects of the citric acid cycle. Biochem Soc Symp **54:** 173-182.

Stoops JK, Awad ES, Arslanian MJ, Gunsberg S, Wakil SJ, Oliver RM (1978) Studies on the yeast fatty acid synthetase: Subunit composition and structural organization of a large multifunctional enzyme complex. J Biol Chem **253:** 4464-4475.

Stoops JK, Wakil SJ (1978) The isolation of the two subunits of yeast fatty acid synthetase. Biochem Biophys Res Commun **84:** 225-231.

Stoops JK, Wakil JS (1980) Yeast fatty acid synthetase: Structure-function relationship and nature of the beta-ketoacyl synthetase site. Proc Natl Acad Sci USA **177:** 4544-4548.

Suss KH, Arkona C, Manteuffel R, Adler K (1993) Calvin cycle multienzyme complexex are bound to chloroplast thylakoid membranes of higher plant *in situ*. Proc Natl Acad Sci USA **90:** 5514-5518.

Terzaghi W (1986) Metabolism of Tween-fatty acid esters by cultured soybean cells. Plant Physiol **82:** 780-786.

Terzaghi W, Roth j, Keim P, Honeycutt RJ, Lark KG (1985) A somatic genetic system for soybean. *In* Freeling, ed, Plant Genetics, Alan R Liss, Inc., New York, pp 771-789.

Topfer R, Martini N (1994) Molecular cloning of cDNAs or genes encoding proteins involved in *de novo* fatty acid synthesis in plants. J Plant Physiol **143:** 416-425.

Towler DA, Gordon JI, Adams SP, Glaser L (1988) The biology and enzymology of eukaryotic protein acylation. Ann Rev Biochem **57:** 69-99.

Vagelos RP (1971) Regulation of fatty acid biosynthesis. Curr Top Cell Regul **4:** 119-166.

Vagelos RP (1974) Biosynthesis of saturated fatty acids. *In* TW Goodwin, ed, MIT International Review of Science. Biochemistry of Lipids, Vol 4, Butterworth, London, pp 100-140.

Vick BA, Zimmerman DC (1984) Biosynthesis of jasmonic acid by several plant species. Plant Physiol **75:** 458-461.

Voelker TA, Worrell AC, Anderson L, Bleinbaum J, Fan C, Hawkins DJ, Radke SE, Davies HM (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. Science **257:** 72-74.

Wakil S (1989) Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry **28:** 4523-4530.

Zenson R,Husmann H, Schneider R, Peine T, Weiss H (1992) *De novo* synthesis and desaturation of fatty acids at the mitochondrial acyl carrier protein, a subunit of NADH:ubiquinone oxidoreductase in *Neurospora crassa*. FEBS Lett **310:** 179-181.

Chapter 2

The protein purification and cDNA cloning of the predominant acyl carrier protein isoform in *Arabidopsis* leaves

Abstract

The predominant ACP isoform found in *Arabidopsis* leaf tissues (the leaf major isoform [LMI] ACP) was purified to homogeneity from *Arabidopsis* leaf tissue. The sequence of the first 25 amino acid residues of the amino-terminus of the mature LMI ACP were determined and used to identify a cDNA clone corresponding to this ACP isoform from the *Arabidopsis* EST cDNA collection. The identity of the LMI ACP cDNA was confirmed by the co-migration of the *Escherichia coli* (*E. coli*) expressed LMI cDNA sequence with the endogenous LMI ACP on native polyacrylamide gels. Although the LMI ACP is the most abundant ACP isoform in leaves, LMI ACP is also found in other green tissues and mature seeds. As was determined by immuno-blot and Northern blot analysis, the LMI ACP was present in extracts made from several green tissues and mature seeds, but was not detectable in roots. Although the deduced amino acid sequence of the mature LMI ACP shared a high degree of similarity with the previously cloned *Arabidopsis* ACPs, the LMI contained several structural features that distinguished it from other *Arabidopsis* ACP isoforms.

Introduction

Acyl carrier protein (ACP) is an essential protein cofactor involved in fatty acid synthesis (Ohlrogge, 1987). This protein acts as a scaffold which shuttles the growing acyl-chain between the various enzymes in the fatty acid synthase complex. All ACPs pocess a highly conserved amino-acid sequence surrounding a serine residue to which the phosphopantetheine prosthetic group is attached. The growing acyl-chain is covalently attached to this prosthetic group through a thioester linkage. The animal and yeast ACPs are present as domains with in type I FAS multifunctional polypeptide complexes (Wakil et al., 1983), where as plant and bacterial ACPs exist as soluble proteins with molecular weights of approximately 10,000 and pI's of around 4 (Ohlrogge, 1987).

In plants, the plastid localized ACP has been the most well characterized component of the FAS complex. Plastid ACP proteins and genes have been isolated from a number of plant species (von Wettstein-Knowles et al., 1994 and references within). Of the known plant ACPs, all show a high degree of amino acid similarity, especially in the region surrounding the prosthetic attachment site (Ohlrogge, 1987).

Plant species have been shown to express multiple isoforms of the plastic localized ACP (Ohlrogge & Kuo, 1985). The number of plastid ACP isoforms present differ not only between plant species, but also between different tissues within a given plant species. Spinach contains two different plastid ACP isoforms in leaves, but only one ACP isoform in roots and seeds (Ohlrogge & Kuo, 1985). The multiple isoforms of plastid ACP have been shown to be coded for by small nuclear gene families in many plant species (von Wettstein, 1994). While the different plastid ACP genes clearly correspond to proteins with

different peptide sequences, evidence from the sequencing of multiple ACP cDNAs from *Brassica napus* showed that some individual ACP isoforms are coded for by multiple genes (Safford et al., 1988).

The functional significance of multiple plastid ACP isoforms is not known. However, in leaf tissue, one possible role for multiple ACP isoforms may be to direct fatty acids towards use in either microsomal or chloroplast glycerolipid synthetic pathways (Roughan and Slack, 1982). The majority of the leaf membrane lipids are synthesized through a microsomal localized glycerolipid synthetic pathway. Fatty acids are directed towards this pathway through the action of long chain acyl-ACP thioesterases which cleave the mature fatty acids from the ACP backbone. The free fatty acids then move across the plastic membrane where they are rapidly converted to acyl-CoAs which serve as substrates for microsomal localized acyltransferases. In addition to the microsomal glycerolipid pathway, the leaves of certain plant species such as *Arabidopsis* and spinach, synthesize a significant amount of their glycerolipids through a chloroplast localized pathway. In these plants, long chain acyl-ACPs serve as substrates for chloroplast localized acyltransferases which transfer fatty acids to the sn-1 and sn-2 position of glycerol backbones (Browse and Sommerville, 1991). It has been proposed that specific ACP isoforms may serve as preferred acyl-ACP substrates either for acy-ACP thioesterases which would direct fatty acids towards microsomal glycerolipid pathways or for plastic localized acyl-ACP acyltransferases. The results from *in vitro* studies suggested that in spinach leaves, the ACP I isoform was the preferred substrate for acyl-ACP thioesterases, while the ACP II isoform served as a better substrate for the chloroplast local ized glycerol-3 -phosphate acyltransferase (GPAT) (Guerra et al., 1986). However, although *in vivo* measurements of acyl-ACP pools in

spinach leaves showed that both ACP I and ACP II participated equally in the initial reactions of fatty acid synthesis, *in vivo* evidence does not exist to indicate whether either plastic ACP isoform is preferred over the other by either GPAT or any of the thioesterases (Post-Beittenmiller et al., 1991).

Arabidopsis thaliana provides a particularly attractive system to study the role of the multiple ACP isoforms in plants. Previous work has shown that *Arabidopsis* contains at least four and as many as six different plastid ACP isoforms, some of which are expressed constitutively in all tissues while others appear to be expressed in a tissue specific manner (Hlousek-Radojcic et al., 1991). The genes coding for three different *Arabidopsis* ACP isoforms have previously been cloned (Post-Beittenmiller et al., 1989; Lamppa et al., 1991; Hlousek-Radojcic et al., 1991) and appear to be expressed constitutively in all tissues (Hlousek-Radojcic et al., 1991).

Arabidopsis leaves have been shown to contain at least 5 different isoforms of ACP (Hlousek-Radojcic et al., 1991). One ACP isoform, designated as the leaf major isoform (LMI) ACP, was present at significantly higher levels than the other ACP expressed in leaves and appeared to be expressed in a leaf specific manner.

The work reported here will describe the protein purification of the leaf major ACP isoform and the subsequent identification of the corresponding cDNA clone. Furthermore, patterns of expression will be shown by immunoblot and Northern blot analysis.

Materials and Methods

(Unless otherwise indicated, all procedures were done at 4°C.)

Purification the leaf major ACP isoform (LMI)

Two hundred gms of *Arabidopsis thalina* leaf material, which was previously frozen in liquid nitrogen and stored at -80°C, was homogenized in 500 ml of 50 mM sodium phosphate pH 6.8, 1 mM DTT using a Brinkmann polytron. The homogenate was centrifuged at 6,500 RPM in a Sorval GS-3 rotor for 20 minutes and the supernatant was filtered through miracloth and collected. Ammonia sulfate was added to the supernatant to 60% saturation and stirred for one hour. The $(NH_4)_3SO_4$ precipitate was pelleted by centrifugation at 10,000 g for 20 minutes. The supernatant was collected and adjusted to 5% trichloroacetic acid (TCA). After stirring for 1 hour, the TCA precipitate was collected by centrifugation at 10,000 g for 20 minutes. The TCA pellet was then resuspended in 50 ml of 100 mM Tris pH 9.0, 100 mM dithiolthrietol (DTT). The resuspended pellet solution was diluted with H_2O until the conductivity was equal to that of a 100 mM NaCl solution. DTT was added to a final concentration of 10 mM and the solution was stirred for 1 hour. After centrifugation at 10,000 g for 10 minutes to remove any insoluble material the solution was applied at 0.833 ml/minute to a 25 ml DEAE cellulose column (DE52, Whatmann) equilibrated with 10 column volumes of 10 mM 2-(N--morpholino) ethanesulfonic acid (MES) pH 6.1, 10 mM DTT at 0.83 ml/minute. After washing with 2 column volumes of 10 mM MES pH 6.1, 10 mM DTT. protein was eluted from the column using a linear gradient of 0 to 500 mM NaCl in 10 mM MES pH 6.1, 10 mM DTT. Fractions containing ACP were identified using the *E. cold* acyl-ACP synthetase assay as described by Kuo and Ohlrogge (1984). Pooled fractions containing the LMI ACP were further purified on a

Mono-Q HR5/5 FPLC column (Pharmacia) equilibrated with 20 mM Tris pH 7.5. Protein was eluted from the column with a O to 300 mM $MgCl_2$ gradient in 10 mM Tris pH 7.5 at a flow rate of 1.5 ml/minute. Protein elusion was monitored by absorbance at 225 nm and individual protein elution peaks were collected by hand. Fractions were analyzed for ACP as described above.

***E. coli* Expression of LMI ACP**

Polymerase chain reaction (PCR) was used to facilitate the cloning of the mature LMI ACP sequence into the pET3D *E. coli* expression vector (Studier et al., 1990). For the LMI expression construct containing two alanine residues at the amino terminus, a forward primer (JO35) with the following sequence was synthesized: CATGCCATGGCCGCGAAAGCAGAGACGGT. For the LMI expression construct that contained a single alanine residue the amino terminus, the forward primer; CATGCCATGCGAAAGCAGAGACGGTGCA was used. Both primers contained *NcoI* sites at the 5' end of each primer. The reverse primer in both cases was designed from the SP6 promoter contained in the cDNA vector. The mature LMI sequences were amplified with the Pfu DNA polymerase (Stratagene, La Jolla, CA) as described by Mullis and Falloona (1987) using thirty cycles as follows: 1 min at 94.C, 2 min 55°C, 2 min 72°C. The PCR products were first treated as described by Crowe et al. (1990) and were then digested with *BamHI* and *NcoI* and subcloned into pET3D. The single and double alanine LMI expression constructs were designated as p1-ala and p2-ala respectively. Both DNA constructs were sequenced as described by Sanger et al. (1977) and no PCR generated errors were detected in either construct. These constructs were transformed into the *E. coli* strain, BL21(DE3) (Studier et al., 1990).

Twenty-five ml cultures of BL21(DE3) cells transformed with the LMI p1-ala and p2-ala constructs, which were started from overnight cultures, were grown at 37°C in Luria broth containing 50 mg/ml ampicillin to a final optical density of 0.6 measured at 600 nm. The expression of the LMI protein expression was then induced by the addition of 0.4 mM isopropyl-B-D-thiogalactopyranoside (IPTG), after which each culture was grown for an additional 4 hours at 37°C. After centrifugation at 6000 g for 10 minutes, the cell pellets were resuspended and homogenized in 5% TCA. The TCA precipitate was centrifuged at 10,000 g for 20 minutes. After resuspending the TCA pellet in 200 mM Tris pH 9.5, 100 mM DTT the ACP was deacylated by incubating the resulting solution at 65°C for one hour. The solution was then adjusted to a final concentration of 5% TCA and incubated on ice for 20 minutes. The TCA pellet was collected as described above. The pellet was washed with 1% TCA and resuspended in 50 mM N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH pH 7.8, 5 mM DTT. Insoluble material was then removed by centrifugation at 10,000 g at 4°C for 20 minutes.

Native PAGE and Immnuoblot analysis

For immunoblot analysis, ACPs from *Arabidopsis* tissues were extracted as described for the extraction of the *E. coli* expressed ACPs except that approximately 1 gm of each tissue was ground to a fine powder in liquid nitrogen prior to the homogenization in 5% TCA. All ACP samples were fractionated by native 15% PAGE as described by Post-Beittenmiller et al. (1991). Prior to electrophoresis, samples were treated with either 5 mM DTT or 5 mM N-ethylmaleiamide (NEM). The protein was blotted to nitrocellulose filters and ACPs were detected using antibodies to spinach ACP I as described by Post-

Beittenmiller et al. (1991). Native gels were silver stained as described by Merril (1990).

RNA Extraction and Northern Blot Analysis

All aqueous solutions used in RNA preparations were treated with 0.1% diethylpyrocarbonate as described by Sambrook et al. (1989). Fresh tissue was harvested and immediately frozen in liquid nitrogen and stored at -80°C until proccessed. The frozen tissue was ground in liquid nitrogen to a fine powder. Three and one half ml of homogenization buffer at 80°C (0.2 M sodium borate, 30 mM [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), 1% SDS, 1% deoxycholate, 2% polyvinylpyrrolidone 40,000, 10 mM DTT) was added to one gm of frozen ground tissue. One volume of 1:1 (v/v) phenol: chloroform was added immediately to each sample and the mixture was vortexed for several seconds. Phases were seperated by centrifugation at 10,000 g for 20 minutes at room temperature and the aqueous phase was extracted twice with one volume of chloroform. The final aqueous fraction was adjusted to 2M LiCl and incubated on ice overnight. The LiCl precipitate was then collected by centrifuging at 10,000 g for 20 minutes and the pellet was washed one time with 2 M LiCl. The washed LiCl pellet was resuspended in 500 µl of H_2O and reprecipitated by adding 1/10 volume of 2M potassium acetate pH 5.5 and two volumes of ethanol. The ethanol precipitate was pelleted at 10,000 g for 20 minutes and resuspended in 500 µl of H_2O and stored at -20°C. Twenty µg of total RNA was then fractionated on formaldehyde gels and blotted to Zeta-Probe nylon membranes (BioRad, Richmond CA) as described by Sambrook et al. (1989). The membranes were prehybridized overnight at 42°C in 5XSSC, 10X Denhardt's solution, 0.1% SDS, 0.1 M potassium phosphate pH 6.8, 100 µg/ml

salmon sperm DNA. The membranes were then hybridized overnight in 5XSSC, 10X Denhardt's solution, 0.1 M potassium phosphate pH 6.8, 100 μg/ml salmon sperm DNA, 10% dextran sulfate, 30% formamide with 1 x 10^6 CPM / ml random primer ^{32}P-dCTP labelled probe. The blots were washed 3 times at 65°C in 0. 1XSSC, 0.1% SDS.

Results and Discussion

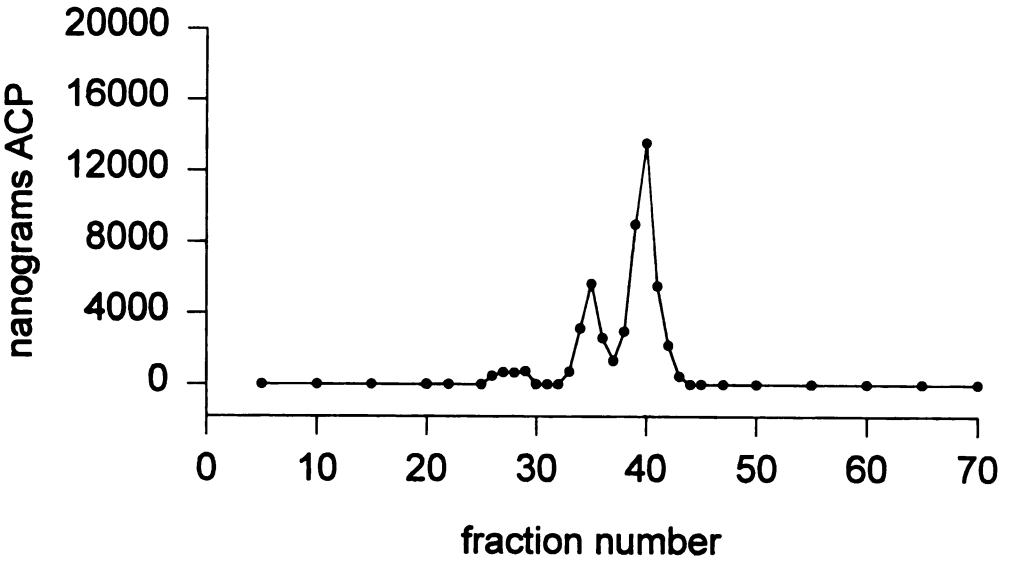
Purification of the *Arabidopsis* LMI ACP

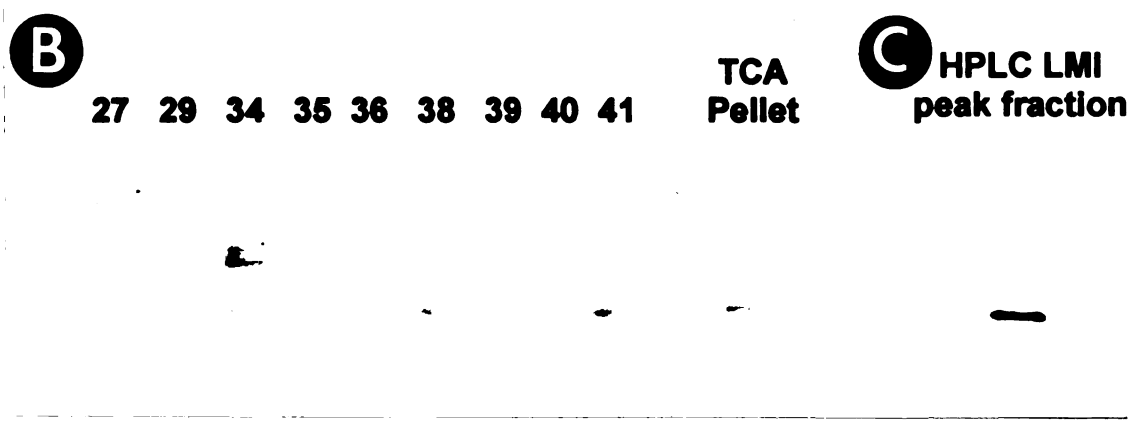
The pattern of ACP isoform expression was extensively characterized in leaf, seed, and root tissue from *Arabidopsis* (Hlousek-Radojcic et al., 1991). These studies showed that *Arabidopsis* leaves contained at least five different isoforms of ACP. One leaf ACP, the leaf major ACP isoform (LMI), was shown to be the predominant ACP in leaves but undetectable in root and seed tissues. Due to this apparant leaf specific pattern of expression of the LMI ACP (Hlousek-Radojcic et al., 1991) and the hypothesis that different leaf ACPs may be involved in directing fatty acids towards different metabolic pathways (Guerra et al.,1986), we became interested in characterizing this particular *Arabidopsis* ACP isoform.

The *Arabidopsis* LMI ACP isoform was purified to homogeneity after two rounds of anion-exchange chromatography. The crude 5% trichloroacetic acid (TCA) pellet, containing the total complement of leaf ACPs, was fractionated into three peaks of ACP after elusion with a salt gradient from a conventional DEAE-cellulose column (Fig. 2-1a). Western blot analysis of these peak fractions showed that the majority of the LMI was present in the third peak (Fig. 2-1b). The DEAE-cellulose eluted fractions containing the LMI ACP were then applied to a mono-Q anion-exchange FPLC column from which the LMI ACP

Figure 2-1. Purification of LMI ACP from *Arabidopsis* leaf. A) The elution profile of ACPs from a DEAE-cellulose column. ACPs were eluted from the DEAE column using a 0 to 500 mM NaCl gradient. Fractions containing ACP were identified using the *E. coli* acyl-ACP synthtease assay. B) Immunblot analysis of DEAE fractions containing ACP. Aliquots from DEAE fractions were run on 15% native PAGE gels, blotted to nitrocellulose and ACPs were then detected with antibodies to spinach ACP I. C) Silver stained native PAGE gel containing an aliquot of the LMI ACP eluted from the mono-Q FPLC column.

A.


20000
16000
12000
8000
4000
0
nanograms ACP
0
10
20
30
40
50
60
70
fraction number


B
27
29
34
35
36
38
39
40
41
TCA
Pellet
C
HPLC LMI
peak fraction

eluted in a single fraction. The mono-Q eluted LMI ACP was purified to homogeneity as demonstrated by Western blot analysis and silver staining of native PAGE gels (Fig. 2-1c).

Protein Sequencing and cDNA Identification

The purified LMI ACP was subjected to amino-terminal protein sequence analysis. Approximately 100 pmoles of LMI ACP was applied to a Applied Biosystems 477A Protein Sequencer. The first 25 amino acids of the mature LMI ACP amino-terminus were identified through this analysis (the underlined amino acid sequence, Fig. 2-2). Initial attempts to clone a cDNA corresponding to the LMI ACP using PCR approaches were unsuccessful. However, a corresponding ACP cDNA clone containing this 25 amino acid sequence was identified from the Arabidopsis EST cDNA collection at Michigan State University (Newmann et al., 1994). Four EST cDNA clones (EST IDs: T45818, T43755, T44265, T43443) shared 100% identity with the first 25 amino acids of the mature LMI ACP. Clone T45818 was obtained from the *Arabidopsis* Biological Resource Center at Ohio State University and the entire cDNA sequence was determined (Fig. 2-2).

Confirmation of the identity of the Putative LMI ACP cDNA

Although the amino-terminal sequence was identical to the deduced sequence from the ACP cDNA identified from the EST collection, further confirmation was needed to prove that the cDNA corresponded to the LMI ACP. Therefore, the cDNA sequence corresponding to the deduced mature ACP was expressed in E. coli and its mobility was compared to the mobility of the endogenous LMI ACP from a leaf extract on native poly acrylamide gels. Native

```
  1 NCNTGNCCGNTCCGGAATTCCNTGGTCGACCCACGCGTCCGTTTTTTTTTTGGGTCTCTG

  1                                 M  A  S  L  S  T  T  S  L  S    10
 61 AGTTGTGTATTGAGCTTCATCTCCTTCAAATGGCTTCCTTATCAACCACTTCCCTAAGCT  120

 11 F  K  A  P  S  T  T  I  S  Q  V  L  R  K  A  S  S  S  Q  S    30
121 TCAAAGCTCCGTCCACCACAATTTCACAGGTTTTAAGGAAAGCCTCAAGTTCTCAGTCTG  180
                                                        ▼
 31 V  T  F  G  R  F  T  S  S  T  K  S  L  R  L  Q  I  S  C  A    50
181 TNACCTTTGGTCGTTTCACGTCTTCAACAAAGAGCCTTCGTCTTCAAATCAGTTGTGCCG  240

 51 A  K  A  E  T  V  Q  K  V  S  D  I  V  K  E  Q  L  A  L  A    70
241 CGAAAGCAGAGACGGTGCAGAAAGTGAGTGACATTGTAAAAGAACAATTGGCTTTAGCTG  300

 71 A  D  V  P  L  T  A  E  S  K  F  S  A  L  G  A  D  S  L  D    90
301 CTGATGTTCCGCTCACTGCTGAATCCAAGTTCTCTGCTCTTGGTGCCGATTCTCTCGACA  360

 91 T  V  E  I  V  M  A  L  E  E  K  F  N  I  S  V  E  E  S  D   110
361 CCGTGGAGATAGTGATGGCGTTGGAGGAAAAGTTTAACATAAGTGTGGAGGAATCTGATG  420

111 A  Q  N  I  T  T  I  L  E  A  A  D  L  I  E  D  L  V  Q  K   130
421 CTCAAAACATTACGACCATCCTAGAGGCGGCTGATTTGATAGAGGACCTTGTTCAAAAGA  480

131 K  P  A  A  E  T  S                                          137
481 AACCCGCGGCGGAAACTTCCTGAACCGGCTGTCTTTTCTTTCCTGACCTAATCTTGCCNT  540

541 CAGAGCGTTTTATTACGGTATATTAGNCCGTTTGTTANTTTTATTTATTTCGGAAGCTAA  600

601 GATTGGGTGTTGACAATGAGACNACTTTGGTACGTTGAGATTGATTCTTTGTGGTGATTG  660

661 GTCTTATGACGAATTCATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  718
```

Figure 2-2. Nucleotide and deduced amino acid sequence of the cDNA clone corresponding to the LMI ACP. The underlined amino acid sequence represents the amino-terminal sequence determined from protein sequencing of the LMI ACP purified from *Arabidopsis* leaf. The closed triangle denotes the position of the transit peptide cleavage site. Amino acid sequence in bold type represents the deduced mature LMI ACP protein sequence. The amino acid sequence in normal type represents the LMI ACP transit peptide.

PAGE has been shown to be very sensitive to small changes in ACP conformation such that a single amino acid change can significantly alter its mobility (Hlousek-Radojcic et al., 1991).

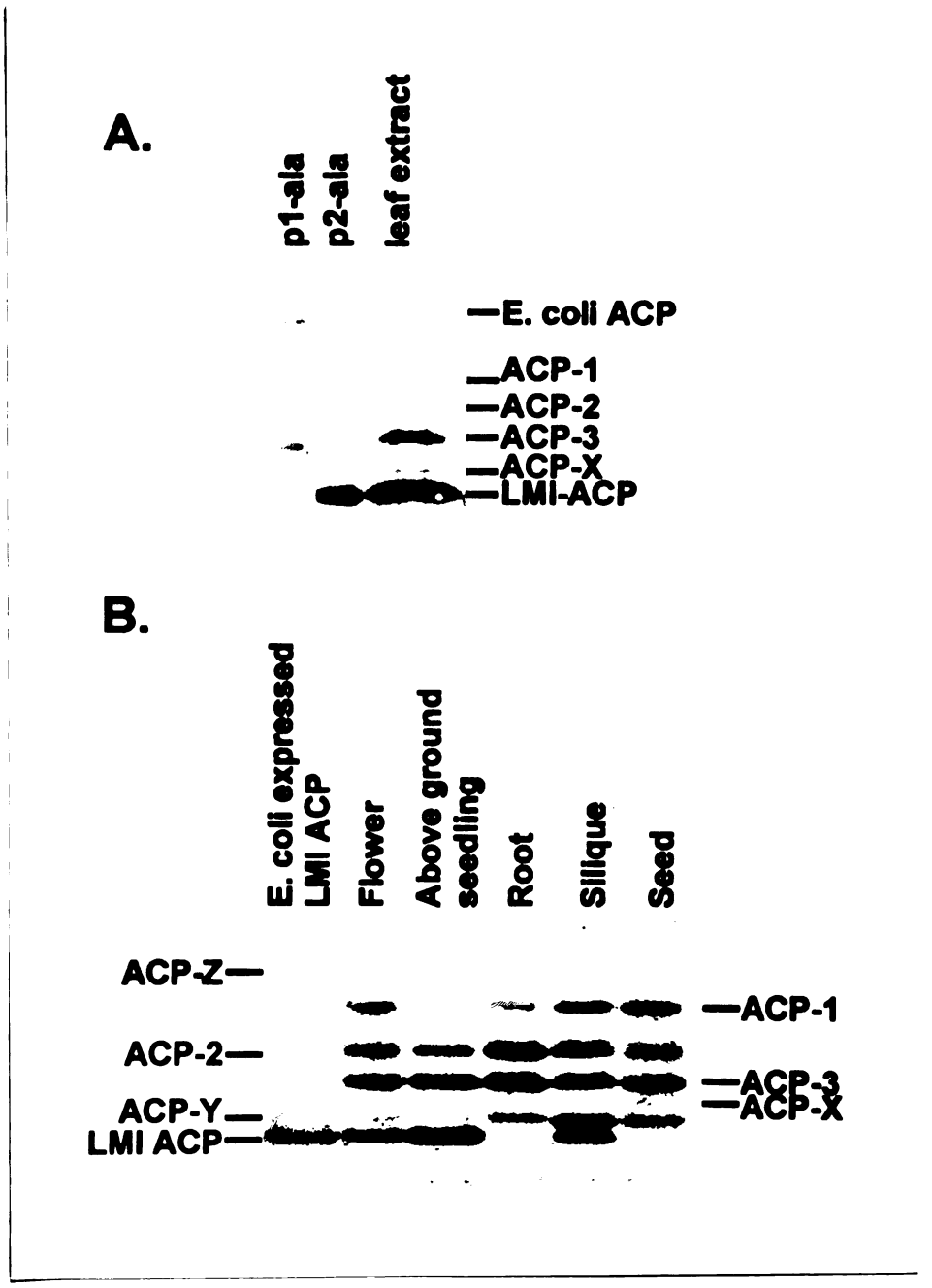
However, because the results from the amino-terminal sequencing was ambiguous as to whether the amino-terminus of the mature LMI ACP contained one or two alanine residues, two PCR derived DNA sequences encoding mature LMI ACPs containing either one or two alanine residues at their amino termini were expressed in *E. coli*. These constructs were designated as p1-ala and p2-ala respecitively.

The mobility of each of the expressed proteins was compared to that of the endogenous LMI ACP from an *Arabidopsis* leaf extract on native poly acrylamide gels. Whereas the *E. coli* expressed p2-ala protein (Fig. 2-3a) co-migrated exactly with the LMI ACP when from an *Arabidopsis* leaf extract (Fig. 2-3a), the mobility of the p1-ala protein differed substantially from the LMI ACP (Fig. 2-3a). The co-migration of the p2-ala *E. coli* expressed LMI protein with the LMI ACP in leaf extracts confirms that the putative LMI cDNA codes for the LMI ACP. Furthermore, these results also confirm that the cleavage of the LMI ACP transit peptide results in the occurance of two alanines at the amino terminus of the mature LMI ACP.

Tissue Specific Patterns of LMI Expression

The tissue specific expression of the LMI ACP was determined by immuno-blot analysis of protein extracts isolated from various *Arabidopsis* tissues. The LMI ACP was detected only in green tissues; including leaves (Fig. 2-3a), above-ground tissues of young seedlings (Fig. 2-3b), siliques (Fig. 2-3b), and whole flower pods (Fig. 2-3b). The level of LMI protein expression was

Figure 2-3. Immunoblot analysis of E. coli expressed LMI ACP constructs and ACP extracts from different *Arabidopsis* tissues. A) Extracts from Arabidopsis leaf and two E. coli expressed LMI ACP constructs containing either one or two alanine residues at their respective amino-terminus were fractionated on native 15% PAGE gels. B) The E. coli expressed LMI ACP (p2-ala) and TCA extracts made from different Arabidopsis tissues were fractionated on native 15% PAGE gels. In both cases (A) and (B), the gels were then blotted to nitrocellulose and ACPs were identified with antibodies to spinach ACP I.


A.
p1-ala
p2-ala
leaf extract
E. coli ACP
ACP-1
ACP-2
ACP-3
ACP-X
LMI-ACP
B.
E. coli expressed LMI ACP
Flower
Above ground seedling
Root
Silique
Seed
ACP-Z
ACP-2
ACP-Y
LMI ACP
ACP-1
ACP-3
ACP-X

highest in leaf extracts where it represented approximately 70% of the total leaf ACP (Fig. 2-3a). In aboveground tissues of young seedlings, which consist primarily of stems and leaves, the LMI ACP represented approximately 30% of the total ACP content, (Fig. 2-3b). The LMI ACP appeared to be minor ACP isoform in siliques, mature seeds and flower pods where it represented less 10% of the ACP complement in both tissues (Fig. 2-3b). The LMI ACP was not detected in roots (Fig. 2-3b).

The immuno-blot analysis also showed that *Arabidopsis* contained at least five different ACP isoforms. As was reported by Hlousek-Radovcic et al. (1992), three ACP isoforms, which have been identified as ACP-1, ACP-2, and ACP-3, were expressed in all tissues. A yet to be purified ACP isoform (designated ACP-X) also appeared to be expressed in all tissues, however, at a much lower level than ACP-1, ACP-2 and ACP-3 (Fig. 2-3b). Two additional ACPs designated as ACP-Y and ACP-Z appeared to be expressed exclusively in flowers and mature seeds respectively (Fig. 2-3b). The expression level of these two proteins appeared to be fairly low relative to other ACP isoforms detected in their rspective tissues.

The levels of LMI ACP mRNA in different *Arabidopsis* tissues were determined by Northern blot analysis. While LMI ACP mRNA was present in leaves, siliques, and flower buds, it was not detectable in roots (Fig. 2-4). This pattern of expression was consistent with the tissue specific pattern of LMI protein accumulation.

Structural comparisons of LMI ACP with other *Arabidopsis* ACPs

The LMI ACP shared between 56% and 60% amino acid sequence identity with the three known *Arabidopsis* ACP protein sequences and contained

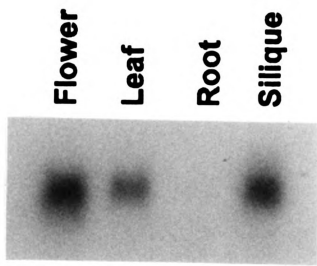
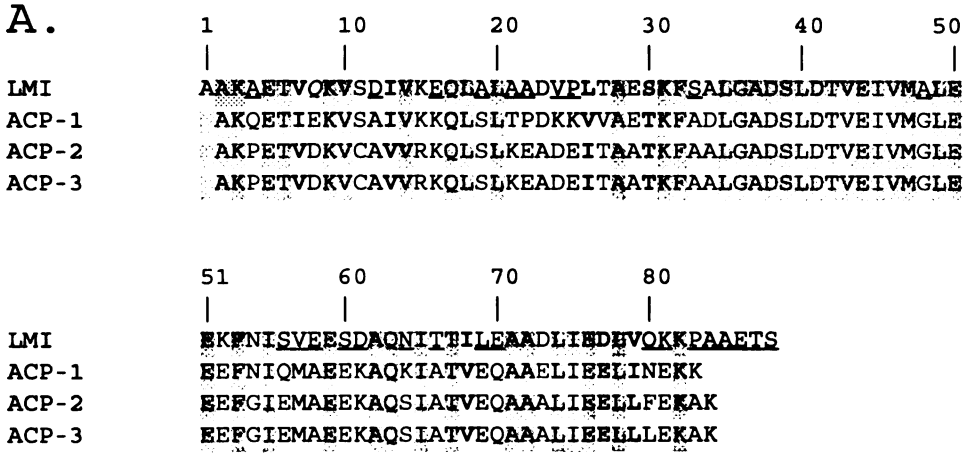


Figure 2-4. Accumulation of LMI-ACP mRNA in different *Arabidopsis* tissue. Approximately 20 μg of total RNA isolated from *Arabidopsis* whole flower, leaf, root and silique tissues was fractionated under denaturing conditions on a 1.2% agarose gel. The RNA was blotted to nylon filters and hybridized with a random primed probe made from the LMI-ACP cDNA.

all of the features associated with a chloroplast localized ACP (i.e. an amino-terminal extension corresponding to a plastic transit peptide, and a phosphopantetheine attachment site). Although the LMI ACP shared significant amino-acid sequence similarity to the three other cloned *Arabdiopsis* ACPs, several features of the LMI ACP distinguished it from these and other plant ACPs. First, the LMI ACP contained two deviations in the concensus sequence corresponding to the phosphopantetheine prosthetic group attachment site (i.e. GADSLTVEIVMGLEEEF) which has been shown to be conserved in all other *Arabidopsis* ACPs and almost all plant ACPs. The first difference occurred at position 48 of the mature protein sequence (Fig. 2-5A) where a glycine has been subsituted for by an alanine. The second change, occurred at position 52 (Fig 2-5A) where a glutamate residue was replaced by a lysine residue. While the exchange at position 48 of a glycine to an a: anine would not be predicted to significantly alter protein structure, the exchange at position 52 of a positively charged amino-acid for a negatively charged amino acid may alter the character of the prosthetic attachment site. These changes were unique to the LMI ACP and have not been observed in any other plant ACPs where the prosthetic group attachment site concensus sequence is strictly conserved.

The LMI ACP also differs from the previously characterized *Arabidopsis* ACP isoforms at the location of the transit peptide cleavage site. Each of the *Arabidopsis* ACPs, including the LMI ACP, shared a significant degree of amino acid sequence similarity in the region surrounding the transit peptide cleavage site (Fig. 2-5B). However, as was determined by the amino termial sequencing of the endogenous LMI ACP and confirmed by the *E. coli* expression of the mature sequence determined from the LMI ACP cDNA, the cleavage site of the LMI ACP transit peptide was shift relative to the other three *Arabidopsis* ACPs

A.



B.

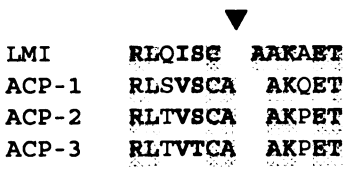


Figure 2-5. A) Alignment of the LMI ACP protein sequence with other *Arabidopsis* plastid ACPs and B) comparisons of the transit peptide cleave sites of other Arabidopsis plastid ACPs. Sequences in bold type represent conserved amino acids. Amino acids that are identical in all ACPs are denoted by shaded characters. Underline amino-acids represent residues that are unique to the LMI ACP. Closed triangle represents the transit peptide cleavage site.

by one amino acid residue towards the amino terminus of the pre-protein.

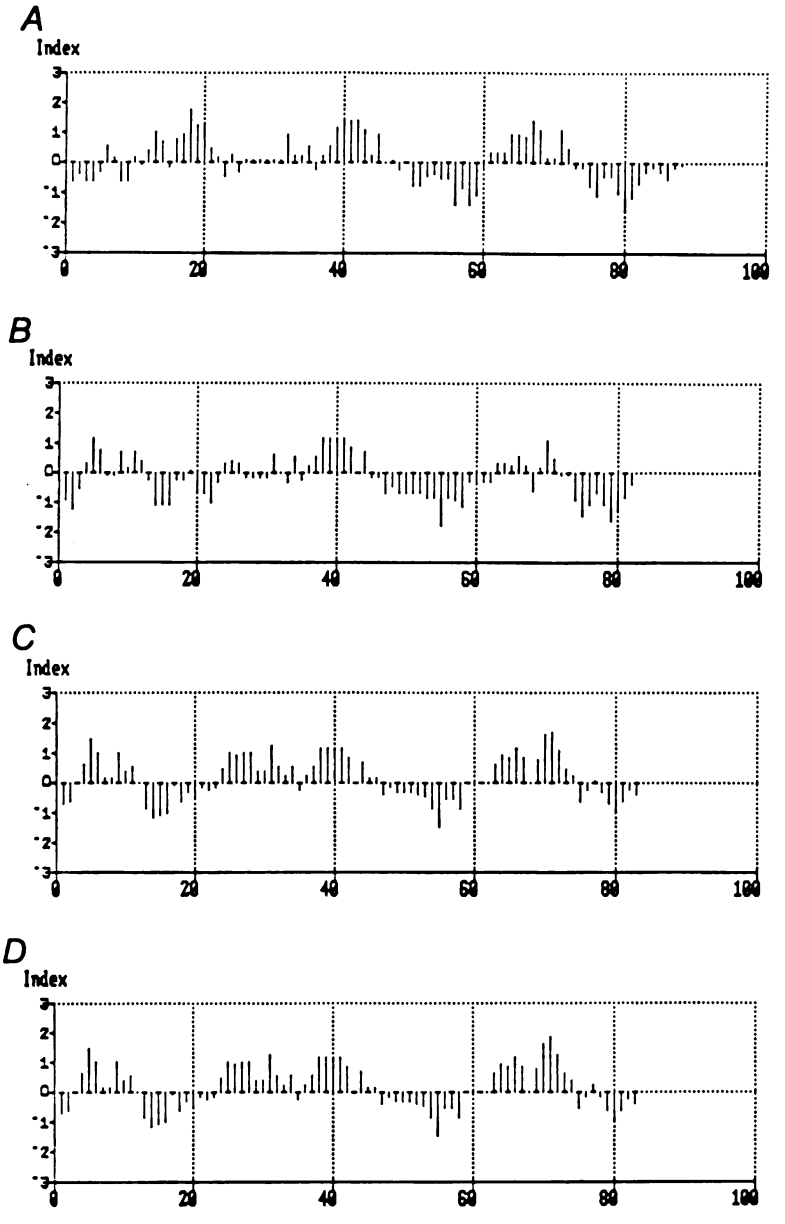
Additionally, hydrophobicity analysis of the four different *Arabidopsis* ACPs showed that the LMI ACP contained a unique stretch of hydrophobic amino acids in the region between amino acid residues 10 and 22 of the mature protein (Fig. 2-6). This region corresponded to a particularly hydrophillic region of the ACP1, ACP2 and ACP3 isoforms. The calculated pI of the LMI ACP also distinguished it from the other *Arabidopsis* ACP isoforms. The pIs of the *Arabidopsis* ACP isoform differed significantly enough to cause the four *Arabidopsis* leaf ACPs to eluted from the DEAE cellulose column as would have been predicted from their calculate pI's. ACP1, which had a the highest calulated pI (i.e. 4.4) was the first ACP isoform to elute from the DEAE column. ACP2 and ACP3, which both have calculated pI's of 4.2, co-elute in the second ACP peak, while the LMI ACP, which had a calculated pI of 3.94, eluted in the final ACP peak.

It is not clear whether these unique features of the LMI ACP have any physiological significance. However, if the hypothesis that specific ACP isoforms act as preferred substrates for specific enzymatic reactions is correct, one would expect that these ACPs would differ structurally from one another. The unique hydrophobic nature of the amino terminal end of the LMI ACP and its more acidic pI may facilitate preferential protein-protein interactions between the LMI ACP and certain fatty acid synthetic enzymes.

Conclusion

The cloning of the LMI ACP is a particularly important achievement because it the first "tissue" specific ACP isoform gene cloned from Arabidopsis. We now have the opportunity to not only alter the expression of the LMI in

Figure 2-6. Hydrophobicity analysis of the Arabidopsis ACP mature protein sequences. The matue protein sequences were subjected to hydophocity analysis using Kyte-Doolittle amino acid classification parameters. The horizontal axis designate amino acid residue position. The vertical axis denotes the degree of hydrophobic / hydrophillic character. Increasing positive values coorelate with increasing hydrophobicity and decreasing negative values coorelate with increasing hydrophillicity. (A) = LMI ACP, (B) = ACP-1, (C) = ACP-2, (D) = ACP-3.


A
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tissues where it is normally found, but it is also possible to express this "tissue" specific ACP isoform in tissues where it is not normally expressed. Such strategies can then be used to determine what, if any, specific role the LMI ACP plays in plant fatty acid synthesis.

Literature Cited

Browse J, Sommerville C (1991) Glycerolipid synthesis: Biochemistry and Regulation. Ann Rev Plant Physiol **42:** 467-506.

Crowe JS, Cooper HJ, Smith MA, Sims MJ, Parker D, Gewert D (1991) Improved cloning efficiency of polymerase chain reaction (PCR) products after proteinase K digestion. Nucleic Acid Res **19:** 184.

Guerra DJ, Ohlrogge JB, Frentzen M (1986) Activity of acyl carrier protein isoforms in reactions of plant fatty acid metabolism. Plant Physiol **82:** 448-453

Hlousek-Radojcic A, Post-Beittenmiller D, Ohlrogge JB (1991) Expression of constitutive and tissue-specific acyl carrier protein isoforms in *Arabidopsis*. Plant Physiol **98:** 206-214.

Lammpa G, Jacks C (1991) Analysis of two linked genes coding for the acyl carrier protein (ACP) from *Arabidopsis thaliana* (*columbia*). Plant Mol Biol **16:** 469-474.

Merril CR (1990) Gel staining techniques. Methods in Enzymology **182:** 477-488.

Ohlrogge JB, KuoTM (1985) Plants have isoforms for acyl carrier protein that are expressed differently in different tissues. J Biol Chem **260:** 8032-8037

Ohlrogge JB (1987) Biochemistry of plant acyl carrier proteins. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 10. Academic Press, New York, pp 137-157.

Post-Beittenmiller MA, Hlousek-Radojcic A, Ohlrogge JB (1989) DNA sequence ofa genomic clone encoding an *Arabidopsis* acyl carrier protein. Nucleic Acid Res. **15:** 7197.

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) In vivo pools of free and acylated acyl carrier proteins in spinach: Evidense for sites of regulation of fatty acid biosynthesis. J. Biol Chem **266:** 1858-1865.

Roughan PG, Slack CR (1982) Cellular organization of glycerolipid metabolism. Ann Rev Plant Physiol **33:** 97-132.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Biology: A laboratory manual, second edition, C Nolan, ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463-5467.

Safford R, Windust JHC, Lucas C, De Silva J, James CM, Hellyer A, Smith CG, Slabas AR, Hughes SG (1988) Plastid-localised seed acyl carrier protein of *Brassica napus* is encoded by a distinct nuclear multigene family. Eur J Biochem **174:** 287-295

Studier FW, Rosenburg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 polymerase to direct expression of cloned genes. Methods of Enzymology **185:** 60-89.

von Wettstein-Knowles P, Knauf V, Ohlrogge JB, Lamppa GA, Safford R, Souciet G (1994) Nomenclature for genes encoding acyl carrier protein (ACP). Plant Molecular Biology Reporter **12:** S31-S34.

Wakil SJ, Stoops JK, Joshi VC (1983) Fatty acid synthesis and its regulation. Annu Rev Biochem **52:** 537-579

Chapter 3

The Characterization of a Mitochondrial Acyl Carrier Protein Isoform Isolated From *Arabidopsis thaliana*.[1]

Abstract

A cDNA clone was isolated from an *Arabidopsis* leaf cDNA library that shared a high degree of protein sequence identity with mitochondrial acyl carrier proteins (mtACPs) isolated from *Neurospora crassa* and bovine heart muscle. The cDNA encoded a 88 amino acid mature protein that was preceded by a putative 35 amino acid presequence. *In vitro* protein import studies have confirmed that the presequence specifically targets this protein into pea mitochondria, but not into chloroplasts. These studies indicated that pea mitochondria were not only able to import and process the precursor protein, but also possessed the ability to acylate the mature protein. The mitochondrial localization of this protein, mtACP-1, was confirmed by western blot analysis. *Arabidopsis* mitochondrial protein extracts contained two cross reacting bands that co-migrated with the mature mtACP-1 and acylated mtACP-1 proteins. The acylated form of mtACP-1 was approximately four times more abundant than the unacylated form and appeared to be localized predominantly in the mitochondrial membrane where as the unacylated mtACP-1 was present mostly in the matrix fraction. Using a chloroplast fatty acid synthase (FAS) system, mtACP-1 was able to function as a co-factor for fatty acid synthesis. However, predominantly short and medium chain fatty acids were produced in FAS reactions supplemented with mtACP-1, suggesting that mtACP-1 may be causing

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[1]The material in this chapter was published previously [Shintani DK, Ohlrogge JB (1994) Plant Physiol 104: 1221-1229]

premature fatty acid chain termination.

Introduction

Acyl carrier proteins (ACPs) are protein co-factors essential in *de novo* fatty acid synthesis (Ohlrogge, 1987). ACPs function to shuttle the elongating fatty acid chain through the reactions of fatty acid synthesis. These proteins are also involved in the initial desaturation and acyl-transfer of 16 and 18 carbon fatty acids (Guerra et al., 1986; Ohlrogge, 1987). In animals and fungi, ACP exists as a sub-domain of the mulitfunctional fatty acid synthase polypeptide (Wakil et al.,1983), whereas in plants and most bacteria, ACPs are soluble low molecular weight acidic proteins (Ohlrogge, 1987).

Although ACPs are most commonly associated with fatty acid metabolism, ACPs are also known to function in the synthesis of membrane derived oligosaccharides (Therisod et al.,1986) and polyketides (Shen et al.,1992). ACP has also been implicated as an acyl-donor involved in the activation of haemolysin, an *E. coli* membrane targeted toxin (Issartel et al., 1991).

Recently, ACPs have been reported to occur in mitochondria of *Neurospora* and bovine heart muscle (Brody and Mikolajczyk, 1988; Sackmann et al., 1991; Runswick et al., 1991). The role of mitochondrial ACP is not well understood. The mtACP has been identified as a component of the NADH:ubiquinone oxidoreductase complex (complex I) of *Neurospora* (Sackmann et al., 1991) and bovine heart muscle mitochondria (Runswick et al., 1991). The acylated form of the mtACP is found to be associated with a matrix domain of the membrane bound NADH:ubiquinone oxidoreductase complex. The function of the mtACP in complex I has not yet been determined. It has also

orted that *Neurospora* mitochondria are capable of *de novo* fatty acid

synthesis (Mikolajczk & Brody, 1990; Zensen et al.,1992), implying that ACP may act as a co-factor of mitochondria FAS. However, because the animal or fungal cytoplasmic FAS is thought to be sufficient to maintain fatty acid metabolism in the cell, it is unclear why this redundant pathway exists. It has been suggested that mitochondrial FAS may be necessary for the synthesis of mitochondria specific membrane lipids such as cardiolipin (Mikolajczyk & Brody, 1990; Zenson et al., 1992). In this regard, Brody et al. (1990) reported that *in vitro* labeling of *Neurospora* mitochondria with [2-^{14}C] malonic acid resulted in the radiolabel accumulating as myristoyl moeities in cardiolipin and phospholipid fractions.

In this report, we describe the isolation and characterization of a putative *Arabidopsis* mtACP cDNA clone. This acyl carrier protein, mtACP-1, shares a high degree of sequence similarity to the *Neurospora* and bovine mtACPs and contains an N-terminal extension that resembled a mitochondrial presequence. The subcellular localization of this ACP and its relationship to fatty acid synthesis are the focus of this research.

Materials and Methods

Screening of the cDNA Library

The mtACP clone, pACP25, was isolated from a λ ZAP (Stratagene, La Jolla, CA) cDNA library constructed from mRNA purified from cold acclimated *Arabidopsis* plants (Lin C & Thomashow MF, 1992). Approximately 60,000 plaques transferred to duplicate nitrocellulose filters (Sambrook et al. 1989) were screened using a 5' end ^{32}P-labelled (Sambrook et al., 1989) 66 nucleotide oligomer corresponding to a region containing the phosphopantetheine binding

site (Hansen, 1987). The filters were prehybridized for 2 hours and hybridized overnight in 5 X SSPE, 1% (w/v) SDS, 0.1% (w/v) blotto at 42°C. The filters were then washed three times for 15 minutes in 5 X SSPE, 1% (w/v) SDS at 42°C and then used to expose x-ray film overnight at -80°C with intensifying screens. pBluescript SK- (Stratagene) plasmids containing desired cDNA sequences were excised from the λ ZAP vector (Stratagene) as described by Short et. al (1988). All further manipulations were performed in pBluescript KS+ as described by Sambrook et al (1989). The identities of positive cDNA clones were confirmed by double stranded dideoxy sequencing with Sequenase 2.0 (U.S. Biochemical, Cleveland, OH) (Sanger et al., 1977).

in vitro Transcription/Translation of _Arabidopsis_ pre-mtACP-I and Spinach pre-cpACP-I precursors.

The DNA sequence corresponding to the mtACP-1 precursor peptide was amplified away from the non-coding sequences of the cDNA clone pACP25 by polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The forward primer (JO107 = 5'-CGGGATCCATGGCACTGAGAAATGC-3') and reverse primer (JO94 = 5'-CGGGATCCGCTAGACATTGGATGATT-3') had *BamH*1 sites engineered into their respective 5' ends to facilitate subcloning into pBluescript II KS+. The PCR reaction contained 2.0 μM each primer, 10 ng of pACP25 template DNA, 2.5 units *Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA), 200 μM each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ 0.01% (w/v) gelatin in a 100 μL volume. The reaction mix was first denatured for 2 minutes at 94°C. The sequence was amplified using 30 cycles of the following thermocyle profile: 1 minute at 94°C, 2 minutes at 55°C, 2 minutes at 72°C. The PCR product was digested with *Bam*HI, subcloned into

pBluescript II KS+, and was designated p0527. p0527 was linearized with *Eco*RV and used as a template to generate pre-mtACP-I transcript using T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN). The spinach cpACP-1 RNA template construct (Schmid & Ohlrogge, 1990) was linearized with *Bam*HI and the pre-cpACP-1 transcript was synthesized using T3 RNA polymerase (Boehringer Mannheim). 10 µg of each precursor transcript was translated in the presence of ^{35}S-methionine using a rabbit reticulocyte lysate system (Promega, Madison, WI).

Mitochondrial and Chloroplast Protein Import Studies

Mitochondria were purified from 400 g of fourteen day old green pea seedlings (Little Marvel) (Burpee Seeds) grown at 20°C on a 12 hour light/dark photoperiod as described by Fang et al (1987). The intactness of the mitochondria was determined by the latency of cytochrome C oxidase activity after Triton-X-100 treatment (Neurburger et al., 1982).

Mitochondrial protein import reactions were performed as described by Watts et al. (1992) with the following modifications. Mitochondria corresponding to approximately 150 µg of protein were incubated for 1 hour at 25°C with 20 µL of *in vitro* translation product in a final volume of 100 µL 0.3 M sorbitol, 20 mM TES, 1 mM DTT, 0.5 mM EDTA, 2 mM K_2HPO_4, 20 mM KCl, 9 mM creatine phosphate, 5.0 mM methionine, 1 mM NADH, 120 µg/mL creatine kinase, 2 mM ATP, pH 7.2. The import reactions were then incubated on ice for 15 minutes followed by an additional 30 minutes with or without 20 µg/mL proteinase K. PMSF was added to a final concentration of 10 mM and the reactions were incubated on ice for 15 minutes. Intact mitochondria were reisolated by centrifugation at 5000 *g* for 5 minutes through a 1 mL 26% (v/v)

Percoll cushion containing 0.25 M sucrose, 0.1% (w/v) BSA, 10 mM TES pH 7.2. The mitochondrial pellets were washed with 200 µL of 0.25 M sucrose, 0.1% (w/v) BSA, 10 mM TES pH 7.2 and repelleted at 5000 *g* for 5 minutes. The mitochondria were resuspended in 50 µL of 1X SDS-PAGE sample buffer and boiled for 5 minutes. Where noted, the mitochondria were resuspended in 100 mM Tris pH 9.0, 100 mM DTT, incubated at 65°C for 30 minutes and then precipitated with 5% (w/v) TCA. The resulting TCA pellet was then resuspended in 50 µL of 1X SDS-PAGE sample buffer. The mitochondrial extracts were electrophoresed on 15% SDS-polyacrylamide gels and blotted onto nitrocellulose filters (Battey and Ohlrogge, 1990). Radioactivity on the blots was detected and analyzed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Spinach chloroplasts were isolated from spinach leaves as described by Roughan (1987). A 200 µL import reaction mixture containing 20 µL of *in vitro* translation product, 8 mM ATP, 0.33 M sorbitol, 50 mM HEPES/KOH pH 8.0, and 100 µL of chloroplasts was incubated for 45 minutes at 25°C. The reactions were incubated on ice for 30 minutes with or without the addition of 20 µg/mL proteinase K (Boehringer Mannheim). PMSF was added to a final concentration of 10 mM and the reactions were incubated for an additional 15 minutes on ice. Intact chloroplasts were re-isolated by centrifugation at 1,500 *g* for 6 minutes through a 1 mL cushion of 40% (v/v) Percoll in 50 mM HEPES/KOH, pH 8.0, 0.33 M sorbitol. The chloroplast pellet was washed in 50 mM HEPES/KOH pH 8.0, 0.33 M sorbitol and repelleted by centrifuging at 1,500 *g* for 6 minutes. The washed chloroplasts were resuspended in 50 µL 25 mM HEPES, pH 8.0 and incubated on ice for 15 minutes to cause lysis. The lysed chloroplasts were then centrifuged at 14,000 *g* for 10 minutes. The resulting supernatant was analyzed

on a 15% SDS-polyacrylamide gel and blotted onto nitrocellulose as described above. The blot was then used to expose X-ray film.

Expression of pACP25 in *E. coli* and polyclonal antibody production

PCR was used to facilitate the cloning of the mature mtACP-1 sequence into the *E. coli* protein expression vector, pET3a (Studier et al., 1990). The forward primer, JO95 (JO95 = 5'-CGGGATCCATATGTCGCACGATGATCATCTT-3') contained a *Nde* I site designed into the 5' end. The M13 reverse sequencing primer was used as the reverse primer. The mature peptide sequence was amplified by PCR as described above. The PCR product was cloned into the *Eco*RV site of pBluescript II KS+ and then subcloned as a *Nde*I *Bam*HI fragment into pET3a. The resulting plasmid was designated p0914A-10. The induced expression of mtACP-1 from *E. coli* BL21 (DE3) cells carrying the plasmid p0914A-10 by IPTG resulted in the production of an abundant protein with a molecular mass of approximately 18 kD which was easily detected in Coomassie blue stained SDS-PAGE gels. To produce antibodies to this protein, a three liter culture of *E. coli* strain BL21 (DE3) containing p01914A-10 was induced by 0.4 mM IPTG when the culture density reached an optical density of 0.7 at 600 nm. The culture was incubated for an additional 6 hours at 37°C after which the cells were harvested by centrifugation at 6000 g for 20 minutes. The cell pellets were washed in homogenization buffer (50 mM MOPS pH 7.5, 10 mM DTT) and resuspended in 30 mLs of the same solution containing 1 mg of DNase I (Sigma). The cells were lysed in a French pressure cell at 20,000 psi. The lysate was diluted to 100 mL with homogenization buffer and centrifuged at 10,000 *g* for 20 minutes. $(NH_4)_2SO_4$ was added to the supernatant to 60%

saturation and was stirred for 30 minutes. The insoluble material was removed by centrifugation at 10,000 *g* for 30 minutes. TCA was added to the supernatant to a final concentration of 5% (w/v), and was then mixed slowly for 30 minutes. The TCA insoluble material was pelleted at 10,000 *g* for 30 minutes. The TCA pellet was washed with 1% (w/v) TCA and resuspended in 40 mL of 100 mM Tris pH 9.5, 100 mM DTT. The resuspended TCA pellet was incubated at 65°C for 30 minutes to deacylate ACPs. The deacylated ACPs were then precipitated in 5% (w/v) TCA as described above. The washed TCA pellet was resuspended in 40 mL of 50 mM MES pH 6.1, 10 mM DTT. The mitochondrial ACP was separated from *E. coli* ACP on a DEAE cellulose (Whatman) column using a linear gradient of 0 - 0.5 M NaCl in 10 mM MES pH 6.1, 10 mM DTT. Column fractions were analyzed for ACP using an acyl-ACP synthetase assay (Kuo and Ohlrogge, 1984). Fractions containing mitochondrial ACP were pooled, TCA preciptated, and resuspended in 10 mM Tris/HCl pH 7.5. The mtACP was further purified on a Mono-Q HR5/5 column (Pharmicia) using a gradient of 0 - 0.3 M $MgCl_2$ in 10 mM Tris/HCl pH 7.5.

Antibodies were generated by injecting the *E. coli* expressed mtACP-1 that was emulsified with Hunter's TiterMax Research Adjuvant R1 (CytRx Corp., Norcross, GA) into New Zealand white rabbits.

Extraction of *Arabidopsis* ACPs

Arabidopsis leaf ACP extracts were prepared from 10 g of *Arabidopsis* leaves. The leaves were ground in liquid nitrogen and homogenized in 100 ml of 10 mM HEPES pH 7.8, 10 mM DTT using a polytron. The homogenate was centrifuged and the supernatant was TCA precipitated and deacylated as described above. The deacylated *Arabidopsis* leaf ACP extract was

resuspended in 2.5 mL of 10 mM HEPES pH 7.8, 10 mM DTT and stored at -20°C in 500 µL aliquots.

Arabidopsis mitochondria were isolated from 18 day old plants grown at 23°C in a 12 hour photoperiod. Plants were placed in the dark for 12 hours prior to mitochondria isolation. Mitochondria were extensively purified on continuous PVP-40/Percoll gradients as described by Day et al. (1985). The isolated mitochondria contained 266 µg/mL protein. The specific activity of cytochrome C oxidase in this preparation was 77 nmoles/min/mg protein. The mitochondria were 86.5% intact as determined by the latency of cytochrome C oxidase activity before Triton X 100 treatment (Neurburger et al., 1982). The mitochondria were free of chloroplast contamination as determined by chlorophyll content. Mitochondria extracts were deacylated as described above.

FAS Assay

Spinach chloroplast fatty acid synthase (FAS) was purified as described by Guerra et al (1987). Spinach cpACP-1 and *E. coli* ACP were purified as described by Ohlrogge and Kuo (1985) and Rock and Cronan (1980) respectively. FAS assays contained 1 mM NADH, 2 mM NADPH, 1 mM acetyl-CoA, 5 mM β-mercaptoethanol, 100 ug/mL spinach leaf protein, 100 mM Tris/HCl pH 8.0 , 400,000 DPM/mL of 54 µCi/µmole [2-^{14}C] malonyl-CoA. FAS reactions were supplemented with mtACP-1 or cpACP-1 at designated concentrations. The reactions were incubated at 30°C. The reactions were then saponified with 0.1 volumes of 10 M KOH and incubated at 65°C for 15 minutes. 0.2 volumes of 5 M H_2SO_4 and 1 volume of a solution of 1 mM palmitic acid in 1M acetic acid in isopropanol were added to each sample. The saponified fatty acids were then extracted three times with 2 volumes of hexane

and analyzed by scintillation counting. FAS reaction products were dried under N_2 and derivatized in 1 mL of boron trichloride at 90°C for 10 minutes. 1 mL of H_2O was added and the fatty acid methyl esters (FAME) were extracted 3 times with 2 mL of hexane. The FAME were then separated on KC18 reverse phase TLC plates (Whatman, Maidstone, England) developed in acetonitrile:methanol:water (65:35:0.5 vol/vol). The amount of ^{14}C label incorporated into each FAME species was determined by phosphorimaging analysis of the TLC plates.

Results and Discussion

Isolation of a cDNA encoding a mitochondrial form of acyl carrier protein

An oligonucleotide probe derived from a highly conserved amino acid sequence surrounding the ACP phosphopantetheine binding site (Hansen, 1987) was used to screen an *Arabidopsis* leaf cDNA library. Forty positive cDNA clones were isolated in this screening, twelve of which were purified and sequenced. Seven of the twelve cDNAs corresponded to two previously cloned *Arabidopsis* ACP isoforms, ACP-2 (Hlousek-Radojcic et al., 1992) and ACP-3 (Lamppa & Jacks, 1991). The identity of four of the sequenced cDNAs was not conclusively determined. The remaining cDNA clone, pACP25, appeared to encode a novel *Arabidopsis* ACP isoform.

pACP25 contained a 2400 base pair cDNA insert. Restriction mapping and Southern blot analysis using the 66mer probe indicated that the ACP sequence was located in a 950 base pair *BamHi HindIII* fragment located in the 3' end of pACP25. This cDNA fragment was subsequently sequenced and two tandem open reading frames were detected. One open reading frame encoded

a protein that showed a high degree of identity to the *Pseudomonas* enzyme, N-carbamyl-L-amino acid amidohydrolase (Watanabe et al., 1992). The second open reading frame, encoded a protein showing a high degree of identity to ACP. The two reading frames were separated by an *EcoRI* site suggesting that two independent cDNA fragments were could have been inadvertently subcloned into the phage vector during the library construction. Because the two open reading frames appeared to be related to two proteins that have very different functions, it is unlikely that the two open reading frames originated from the same mRNA species.

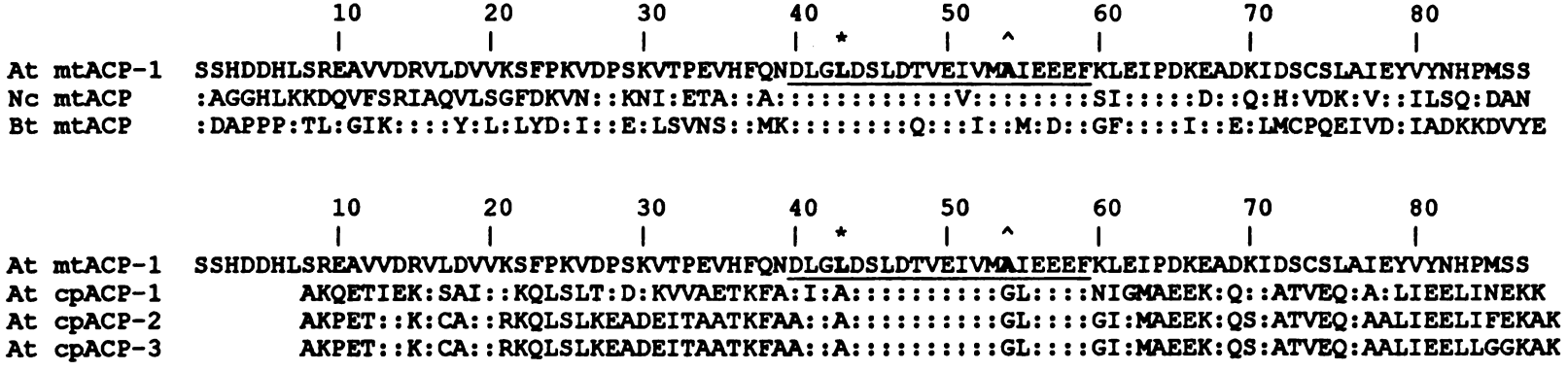
The protein encoded by the second open reading frame of pACP25, designated mtACP-1, contained the phosphopantetheine binding domain which is highly conserved among all ACPs (Ohlrogge, 1987). A putative translation initiation sequence (Heidecker & Messing, 1986) (underlined in Fig. 3-1) surrounded the AUG initiation codon. No polyadenylation site consensus sequence or poly (A) tail was found in the 3' untranslated sequence.

The mature mtACP-1 protein sequence was compared to the *Arabidopsis* cpACP sequences and the known mtACP sequences (Fig 3-2). Although the amino acid sequence corresponding to the region surrounding the prosthetic group attachment site (underlined in Fig. 3-2) was highly conserved among mtACP-1 and all other ACPs, two amino acid changes common to each of the mtACPs were detected in this region. First, at position 44 (denoted by * in Fig. 3-2), a highly conserved alanine seen in all cpACPs was replaced by a leucine in the mtACPs. A second alteration in the conserved sequence of the cpACPs was seen at position 54 (denoted by ^ in Fig. 3-2) where a highly conserved glycine was replaced by an alanine in the mtACP sequences. The region where these changes occur is totally conserved among all known cpACPs sequences

```
                m  a  l  r  n  a  i  l  r  h  l  r  v  p  v  q   16
 1  GAAAGAGACGAAAATGGCACTGAGAAATGCAATTCTTCGTCACCTGAGGGTTCCGGTGCA   60
17    t  l  g  l  n  q  s  k  i  g  f  l  g  t  i  r  s  f  S  S   36
61  AACCCTAGGATTGAATCAGTCTAAAATTGGGTTCCTTGGTACGATCCGGTCATTTTCTTC  120
37    H  D  D  H  L  S  R  E  A  V  V  D  R  V  L  D  V  V  K  S   56
121 GCACGATGATCATCTTAGCAGAGAAGCTGTCGTCGATAGAGTACTCGATGTTGTCAAGAG  180
57    F  P  K  V  D  P  S  K  V  T  P  E  V  H  F  Q  N  D  L  G   76
181 CTTCCCCAAAGTCGATCCCTCTAAGGTGACTCCTGAGGTTCATTTCCAAAACGATTTGGG  240
77    L  D  S  L  D  T  V  E  I  V  M  A  I  E  E  E  F  K  L  E   96
241 ATTAGATAGTTTGGACACAGTGGAGATAGTGATGGCTATTGAAGAGGAATTCAAGCTGGA  300
97    I  P  D  K  E  A  D  K  I  D  S  C  S  L  A  I  E  Y  V  Y  116
301 AATTCCAGACAAAGAAGCTGACAAGATCGATTCTTGCTCTCTCGCCATTGAATACGTTTA  360
117   N  H  P  M  S  S
361 CAATCATCCAATGTCTAGCTAATCGCTGTGTGCTCTCTCATGGTTTCTCCTTCCTTTTTC  420
421 TTGTATGAGGTTCTTCTCTCTTTGATAATGCCATAATGGGATTTGGCAAAAAAATCACTT  480
481 TTATTTGGAGAGCATTGTTATTGACTCTTTTAACGGTTCCAGTTTCTTAAAAAGAAAACC  540
541 TTTTTGGAATTCC
```

Figure 3-1 Nucleotide and deduced amino acid sequence of pACP25. The putative mitochondrial presequence sequence is denoted by the lower case single letter amino acid code. The underlined nucleotide sequence represents the translational initiation consensus sequence (Heidecker and Messing, 1986). The bold upper case single amino acid sequence represents the conserved sequence surrounding the phosphopantetheine attachment site.

Figure 3-2 Alignment of mtACP-1 protein sequence (a) with known mitochondrial ACPs and (b) with the *Arabidopsis* chloroplast ACPs. (:) denote conserved amino acids. * and ^ respectively designate highly conserved alanine and glycine residues seen in all chloroplastic ACPs that have been replaced by leucine and alanine residues (respectively) in the three mitochondrial ACPs. The underlined sequence represents the conserved sequence surrounding the phosphopantetheine attachment site. At mtACP-1 = *Arabidopsis thaliana* mitochondrial ACP; Nc mtACP = *Neurospora crassa* mitochondrial ACP (Sackmann et al. (1991)); Bt mtACP = bovine mitochondrial ACP (Runswick et al. (1991)); At cpACP-1 = *Arabidopsis thaliana* chloroplast ACP-1 (Post-Beittenmiller et al. (1989)); At cpACP-2 = *Arabidopsis thaliana* chloroplast ACP-2 (Lamppa and Jacks (1991)); At cpACP-3 = *Arabidopsis thaliana* chloroplast ACP-3 (Lamppa and Jacks (1991)).


10 20 30 40 50 60 70 80
At mtACP-1 SSHDDHLSREAVVDRVLDVVKSFPKVDPSKVTPEVHFQNDLGLDSLDTVEIVMAIEEEFKLEIPDKEADKIDSCSLAIEYVYNHPMSS
Nc mtACP :AGGHLKKDQVFSRIAQVLSGFDKVN::KNI:ETA::A:::::::::::V::::::::SI:::::D::Q:H:VDK:V::ILSQ:DAN
Bt mtACP :DAPPP:TL:GIK::::Y:L:LYD:I::E:LSVNS::MK::::::::Q:::I::M:D::GF::::I::E:LMCPQEIVD:IADKKDVYE
10 20 30 40 50 60 70 80
At mtACP-1 SSHDDHLSREAVVDRVLDVVKSFPKVDPSKVTPEVHFQNDLGLDSLDTVEIVMAIEEEFKLEIPDKEADKIDSCSLAIEYVYNHPMSS
At cpACP-1 AKQETIEK:SAI::KQLSLT:D:KVVAETKFA:I:A::::::::::GL::::NIGMAEEK:Q::ATVEQ:A:LIEELINEKK
At cpACP-2 AKPET::K:CA::RKQLSLKEADEITAATKFAA::A::::::::::GL::::GI:MAEEK:QS:ATVEQ:AALIEELIFEKAK
At cpACP-3 AKPET::K:CA::RKQLSLKEADEITAATKFAA::A::::::::::GL::::GI:MAEEK:QS:ATVEQ:AALIEELLGGKAK

but is different in mtACP-1 and the bovine and *Neurospora* mtACP sequences. In addition to these specific changes in the highly conserved phosphopantetheine binding domain, when compared to known ACP protein sequences, mtACP-1 shared the highest degree of identity with the mtACPs. The *Neurospora* and bovine heart muscle mtACP protein sequences are 68% and 67% identical to mtACP-1 respectively, whereas the *Arabidopsis* cpACPs share less then 60% identity with mtACP-1. The *Arabidopsis* cpACP sequences share at least 69% identity among themselves (Hlousek-Radojcic et al.,1992); thus the sequence of the mtACP-1 protein appeared to be more conserved with widely divergent organisms of three eukaryotic phylogenetic kingdoms than with cpACPs isolated from the same species, *Arabidopsis*. The alignment of mtACP-1 amino acid sequence with the *Neurospora* and bovine mtACPs sequences suggested that mtACP-1 encoded an 88 amino acid mature protein preceded by a 35 amino acid N-terminal extension. Both the calculated pI of 4.26 and molecular mass of 9,914 daltons were consistent with other ACPs of both chloroplast and mitochondrial origin.

The 35 amino acid N-terminal extension (small case letters in Fig.3-1) shared many of the structural features common to mitochondrial presequences as defined by von Heijne et al (1989). These features included a high degree of alpha helical nature at the N-terminus, hydrophobic amino acids at the C-terminus of the presequence between amino acids 24 and 34, and a highly conserved arginine at position 32. Using these criteria it was hypothesized that the first 35 amino acids of the protein encoded by pACP25 represented a mitochondrial presequence.

***E. coli* Expression of pACP25**

The cDNA sequence encoding the mature mtACP-1 protein was subcloned into the *E. coli* protein expression vector pET3a. mtACP-1 could be clearly separated from the *E. coli* ACP by DEAE-cellulose anion exchange chromatography and was purified to near homogeneity as determined by silver staining of SDS-PAGE gels (data not shown). The *E. coli* mtACP-1 migrated as a 18.0 kD doublet when analyzed by SDS-PAGE. Pure acyl carrier proteins have been reported to sometimes migrate as doublets during PAGE (Jackowski and Rock, 1987). The purified mtACP-1 protein was used to generate polyclonal antibodies in rabbits. The resulting antibodies were highly specific for mtACP-1. Although the antibodies did cross react with other ACP isoforms due to the high degree of similarity shared among all ACP isoforms, these antibodies showed a marked preference for mtACP-1. At a 1:500 dilution, the crude antiserum was sufficient to detect as little as 10 nanograms of mtACP-1 on a Western blot (data not shown).

***In vitro* protein import studies.**

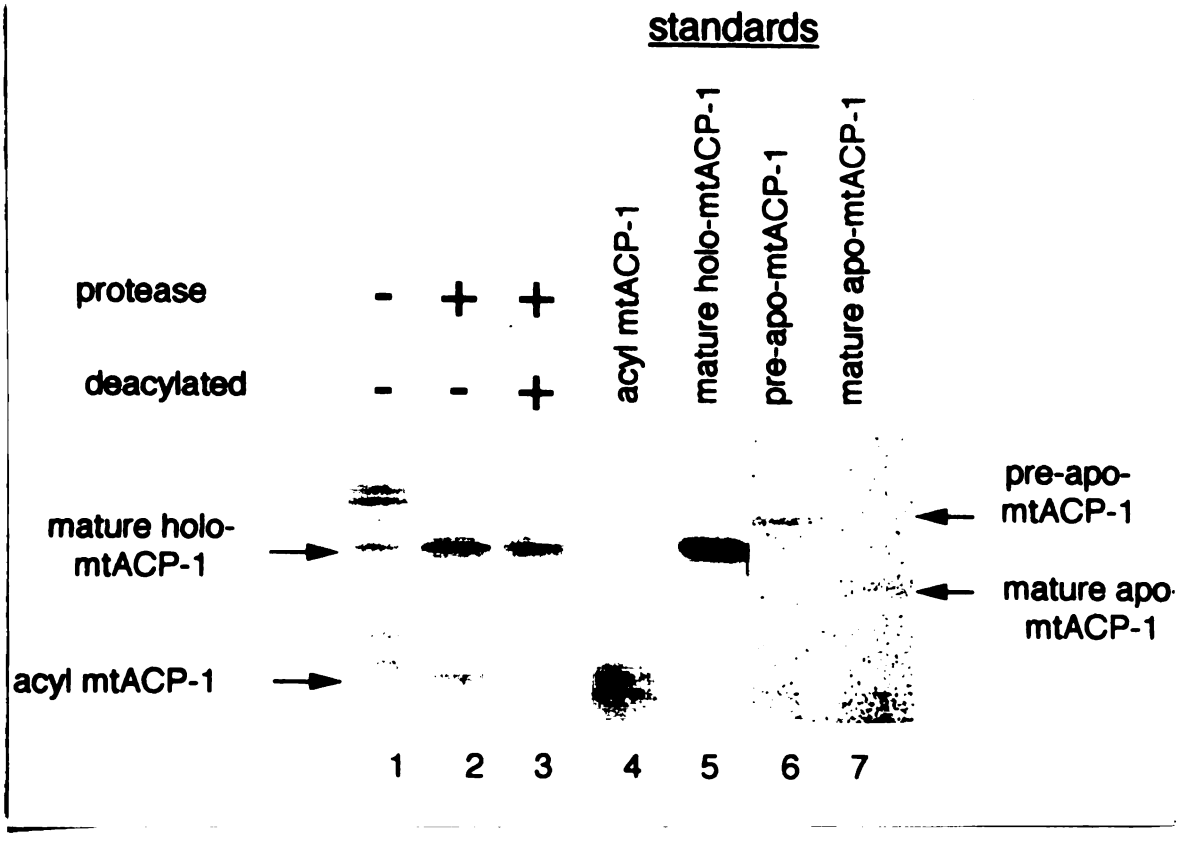
To confirm the targeting specificity of the mtACP-1 presequence, *in vitro* mitochondrial and chloroplastic protein import studies were conducted. The competency of mitochondria and chloroplasts for the import of the mtACP-1 and spinach cpACP-1 precursors was compared. Pea mitochondria and spinach chloroplasts were incubated in the presence of radiolabelled apo-mtACP-1 or spinach apo-cpACP-1 precursor proteins with and without the addition of proteinase K. Proteins imported into the organelles were protected from digestion by exogenously added protease and were detected in mitochondrial or chloroplast extracts by autoradiography. The identities of processed proteins

were determined by mobilities on SDS-PAGE gels relative to mobilities of ACP standards.

The mtACP-1 presequence proved to be sufficient for targeting protein import into pea mitochondria. Although several radiolabelled proteins appeared to be associated with the reisolated mitochondria after incubation with *in vitro* translated apo-mtACP precursor (Fig. 3-3, lane 1), only two proteins, with apparent molecular masses of 18 kD and 12 kD, were protected from protease treatment (Fig. 3-3, lane 2).

The 18 kD radiolabelled protein co-migrated with the mature-holo mtACP-1 standard (Fig. 3-3, lanes 2 and 5) and the 12 kD band appeared to migrate slightly higher than the mature palmitoyl-mtACP-1 standard (Fig. 3-3, lanes 2 and 4). Because the 12 kD band did not migrate exactly with the palmitoyl-mtACP-1 standard, it is possible that the mtACP acylated during the import reaction was composed of a fatty acid of a different chain length since acyl-ACPs are known to migrate on native PAGE differently depending on the fatty acids chain length (Post-Beittenmiller et al., 1990). To confirm that the 12 kD protein did correspond to an acylated form of mtACP-1, post-import mitochondrial extracts were deacylated by treatment with 100 mM DTT at pH 9.0 (Lakin-Thomas and Brody, 1985). Upon deacylation, the 12 kD radiolabelled band disappeared while the presence of the 18 kD band was not affected (Fig. 3-3, lane 3). Thus it appears that the pea mitochondria were not only able to import and process the mtACP-1 precursor, but were also able to acylate the mature mtACP-1 protein. These results contrast with chloroplast protein import experiments where acylated forms of ACP were not detected (Fernandez and Lamppa, 1990a and 1990b). Although *Neurospora* mitochondria have been reported to be capable of de novo fatty acid synthesis

Figure 3-3 *in vitro* protein import of mtACP-1 precursor into pea leaf mitochondria. Mitochondrial protein import studies were performed as described in the Materials and Methods section. The radiolabelled proteins were detected on protein blots using a Molecular Dynamics phosphorimaging system. Lanes 1-4 were derived from images obtained when the background to signal range was set at 10 to 1000 counts. Lanes 6 and 7 were derived from images obtained when the background to signal range was set at 10 to 50 counts. Lane 1, import mixture containing pea mitochondria and radiolabelled mtACP-1 precursor. Lane 2, same as lane 1 but the mitochondria were incubated with proteinase K after import. Lane 3, same as lane 1, but the mitochondria were treated with proteinase K and then deacylated (Lakin-Thomas and Brody, 1985). Lane 4, acyl-mtACP-1 standard that was synthesized *in vitro* from mtACP-1 and ^{14}C-palmitic acid using *E. coli* acyl ACP synthetase (Kuo and Ohlrogge, 1984). Lane 5, mtACP-1 protein standard derived from *E. coli* expressed pACP25 and detected by Western-analysis. Lane 6, radiolabelled mtACP-1 precursor standard synthesized from rabbit reticulocyte lysates and transcripts derived from p0527. Lane 7, radiolabelled apo-mtACP-1 synthesized from rabbit reticulocyte lysate and transcripts derived from p0914A-10.


standards
protease
deacylated
acyl mtACP-1
mature holo-mtACP-1
pre-apo-mtACP-1
mature apo-mtACP-1
mature holo-mtACP-1
acyl mtACP-1
pre-apo-mtACP-1
mature apo-mtACP-1
1 2 3 4 5 6 7

(Mikolajczk and Brody, 1990; Zensen et al., 1992), it is not known if the acyl chain esterified to the mature mtACP-1 is derived from mitochondrial *de novo* fatty acid synthesis or from an acyl-transfer reaction from a preformed acyl group.

Interestingly, no radiolabelled protein was detected in the post-import mitochondrial extract that co-migrated with the mature apo-mtACP-1 standard (Fig. 3-3, lane 7). This suggests that the pea mitochondria are able to very efficiently add the phosphopantetheine prosthetic group to imported mtACP-1 protein. These results contrast with chloroplast protein import studies in which the complete conversion of the apo-cpACP I to the holo form was not seen (Fernandez and Lamppa, 1990a and 1990b). Our results further indicate that the mitochondria may contain holo ACP synthetase (HAS), the enzyme responsible for the addition of the prosthetic group. Elhussein et al. (1988) have reported that HAS activity was associated with both cytoplasmic and plastid cell fractions with relatively little HAS activity being detected in the mitochondria. Although the results shown in figure 3 strongly suggest that the pea mitochondria possess HAS, it is possible that the mtACP precursor was modified prior to import by contaminating cytoplasmic HAS present in the mitochondria preparation. However, it is not likely that the prosthetic group was added during the *in vitro* translation reaction since it is known that the rabbit reticulocyte lysates contain no HAS activity (Linda Savage & Dusty Post-Beittenmiller, unpublished data). In additional control experiments it was shown that the spinach cpACP-1 precursor was not able to direct protein import into the mitochondria (data not shown).

When the mtACP-1 precursor was incubated with spinach chloroplasts, it was able to bind to the outer chloroplast envelope (Fig. 3-4, lane 1) however,

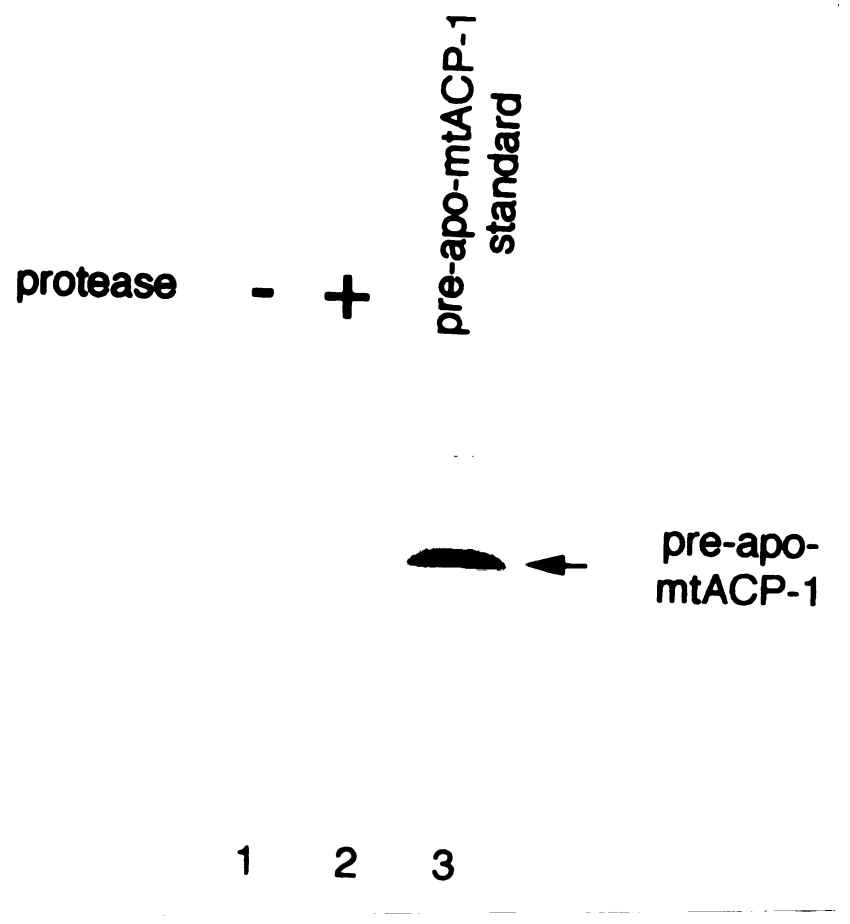


Figure 3-4 *in vitro* protein import studies of the mtACP-1 precursor into spinach chloroplasts. Chloroplast protein import studies were performed as described in the Materials and Methods section. The radiolabelled proteins were detected by autoradiography. Lane 1, import mixture containing spinach chloroplasts and radiolabelled mtACP-1 precursor. Lane 2, same as lane 1 but the chloroplast were incubated with proteinase K after import. Lane 3, radiolabelled mtACP-1 precursor standard synthesized from rabbit reticulocyte lysates and transcripts derived from p0527.

after protease treatment, no chloroplast import had occurred (Fig. 3-4, lane 2). However, as expected, the spinach cpACP-1 precursor was imported and correctly processing by spinach chloroplasts (data not shown).

Sub-cellular localization

To confirm the mitochondrial localization of mtACP-1, Western analysis of *Arabidopsis* leaf extracts was performed. Blots of *Arabidopsis* protein extracts run on 15% SDS-PAGE gels were probed with antibodies specific for mtACP-1. To simplify the number of potential forms of mtACP-1, protein extracts were first deacylated (Lakin-Thomas and Brody, 1985). A 18 kD doublet that cross reacted with the mtACP-1 antibodies was detected in deacylated whole leaf and deacylated purified mitochondria extracts (Fig. 3-5, lanes 1 and 2). These proteins co-migrated with the *E. coli* expressed mtACP-1. The anti-mtACP-1 antibodies weakly cross reacted with proteins that migrated to the known positions of the *Arabidopsis* cpACP isoforms (Battey and Ohlrogge, 1990) in whole leaf extracts (Fig. 3-5, lane 1). Based on the total ACP content of *Arabidopsis* leaf, as estimated from acyl- ACP synthetase, and the level of mACP-1, as approximated from Western blots, we determined that the mtACP-1 may constitute as much as 10% of the total *Arabidopsis* leaf ACP.

In addition to the unacylated form of mtACP-1, intact *Arabidopsis* mitochondria contained a cross-reactive protein that co-migrated with the acyl-mtACP-1 synthesized *in vitro* (Fig. 3-5, lane 3). As estimated from the staining intensity of Western blots, the acylated form of mtACP-1 appeared to be present at approximately 4 times the level of free mtACP-1 in purified mitochondria. This result differs from spinach cpACPs where the free ACP accounted for approximately 60% of the cpACP pool (Post-Beittenmiller et al, 1990). Further

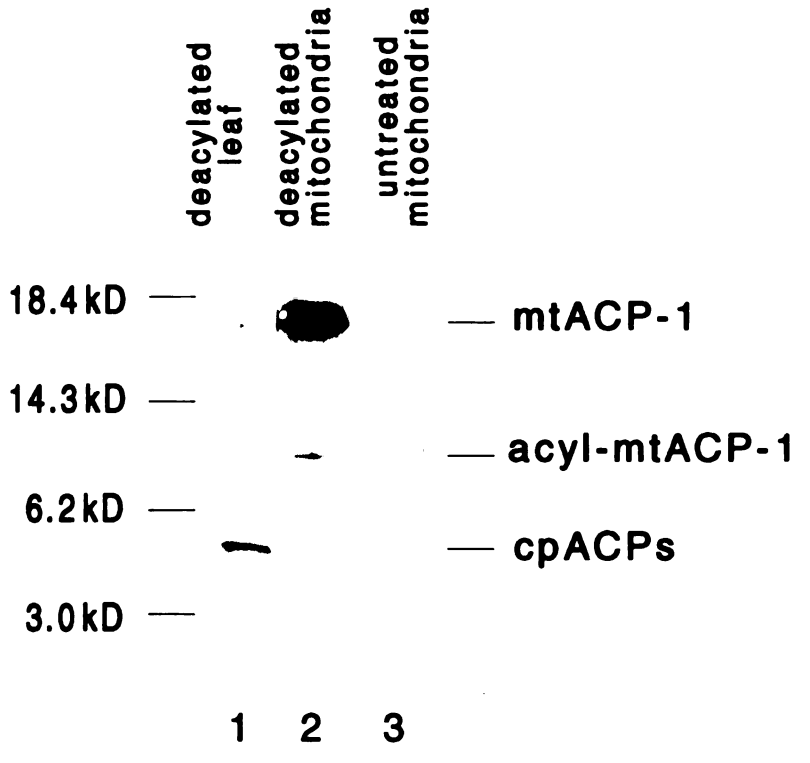


Figure 3-5 Western-blot analysis of *Arabidopsis* leaf sub-cellular fractions. *Arabidopsis* whole leaf and mitochondrial protein extracts were purified as described in the Materials and Methods section. Where noted, the extracts were deacylated by treatment with 100 mM DTT at pH 9.0 for 30 minutes at 65°C. Deacylated leaf protein extract (lane 1), deacylated mitochondrial protein extract (lane 2), untreated mitochondrial protein extract (lane 3).

analysis showed that the acylated form of mtACP-1 was primarily associated with the mitochondrial membrane fraction. The 100,000 g pellet of the lysed mitochondria appeared to contain the majority of the acylated mtACP-1 along with a small amount of free mtACP-1 (Fig. 3-6, lane 2). The free mtACP-1 was present in relatively higher amounts than the acylated form in the 100,000 g supernatant fraction (Fig. 3-6, lane 3). The membrane localization of the acylated mtACP-1 was consistent with the report that the *Neurospora* and bovine mtACPs were only present in the acylated form when associated with the NADH:ubiquinone oxidoreductase membrane complex (complex I) (Sackmann et al.,1991; Runswick et al. 1991), further evidence is necessary to determine if mtACP-1 is also associated with complex I in *Arabidopsis* mitochondria.

Fatty acid synthase (FAS) assays

Although *Neurospora* mitochondria have been reported to be capable of *de novo* FAS (Mikolajczyk & Brody, 1990; Zensen et al., 1992), no direct evidence exists to show that the mtACP functions as a co-factor for fatty acid synthesis. To determine whether or not mtACP-1 was able to act as a co-factor in FAS, *in vitro* chloroplast FAS activities were compared using mtACP-1 and spinach cpACP-1 concentrations ranging from 0.5 μM to 25 μM. The assays were performed using concentrations of chloroplast FAS extracts that would allow linear rates of FAS activity during a 15 minute time period. Although the rates of fatty acid synthesis varied depending on the FAS preparation, mtACP-1 was in all cases able to act as a co-factor for spinach chloroplast FAS when compared to spinach cpACP-1 (Fig. 3-7). These results suggest that despite its structural divergence, mtACP-1 could function as a co-factor for chloroplast FAS.

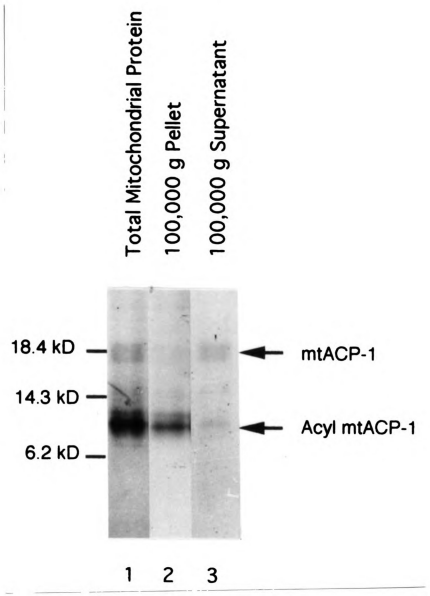


Figure 3-6 Western-blot analysis of mitochondrial membrane and matrix fractions. *Arabidopsis* mitochondria were purified as described in the Materials and Methods section. The mitochondria were lysed by sonication and fractionated by centrifugation at 100,000 g. Unfractionated mitochondria (lane 1), 100,000 *g* pellet from sonicated mitochondria (lane 2), and 100,000 *g* supernatant from sonicated mitochondria.

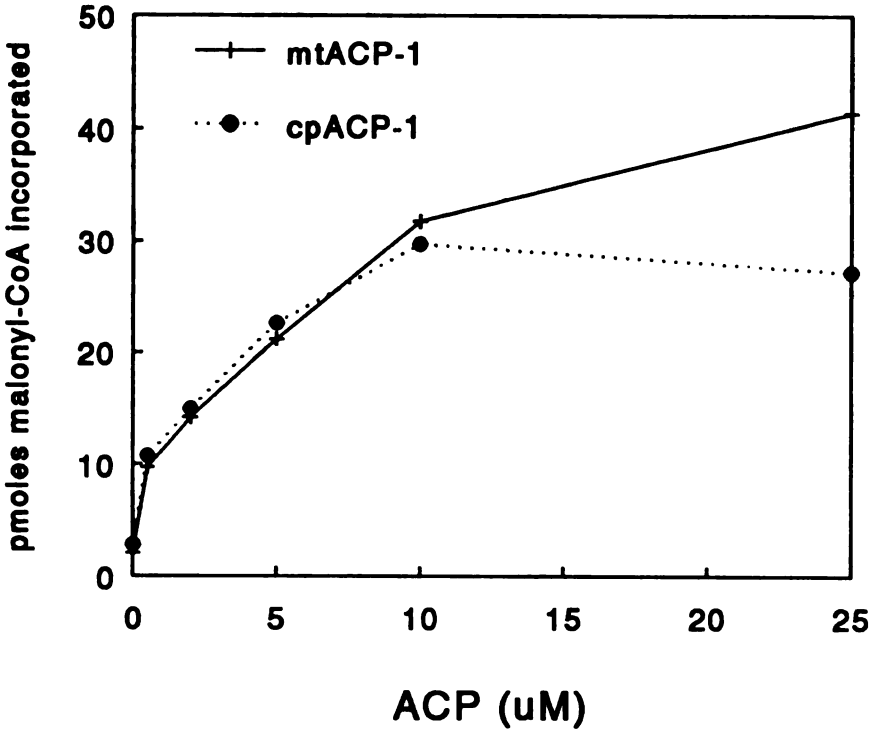


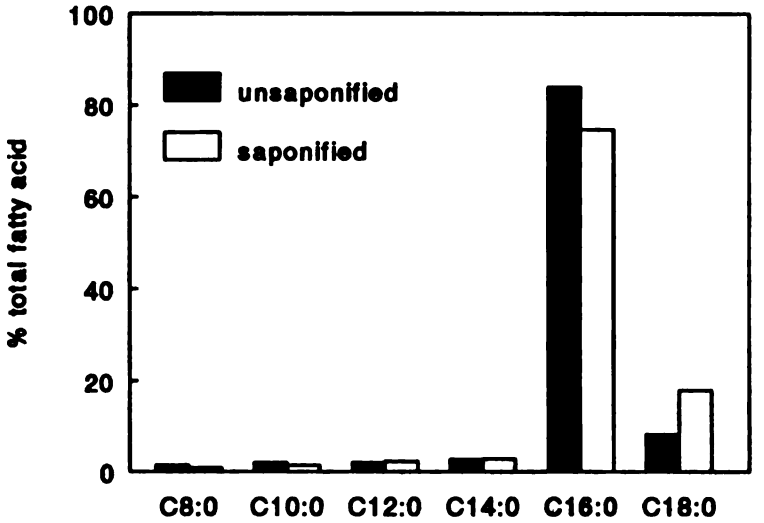
Figure 3-7 Utilization of mtACP-1 and spinach cpACP-1 by chloroplast FAS. 75 µL reactions were incubated for 15 minutes at 30°C. Activity was measured as incorporation of ^{14}C malonyl-CoA into the lipid fraction. Lipid extracted into hexane following saponification and neurtalization of the reaction mixture were counted by liquid scintillation.

The profiles of the fatty acids synthesized by the chloroplast FAS in the presence of mtACP-1 and spinach cpACP-1 were compared. As expected, FAS reactions incubated with spinach cpACP-1 yielded predominately long chain fatty acids (Fig. 3-8A). However, when FAS reactions were supplemented with mtACP-1, short, medium and long chain fatty acids were synthesized (Fig. 3-8B). Caproic acid, lauric acid, myristic acid, and palmitic acid each made up approximately 20% of the total fatty acid, while octanoic and stearic acids each represented approximately 10%. A similar fatty acid profile was observed at mtACP-1 concentrations ranging from 0.5 μM to 5.0 μM (data not shown). To determine if the short and medium chain fatty acids derived from the FAS reactions supplemented with mtACP-1 were present as acyl-ACPs, the reactions were extracted prior to saponification to analyze only free fatty acids (Fig. 3-8A and B). These analysis showed that the short and medium chain fatty acids produced in reactions containing mtACP-1 were not esterified to mtACP-1. The results suggest that the fatty acid products produced on mtACP-1 were prematurely cleaved from the ACP. Although the accumulation of fatty acids of intermediate-chain lengths were also observed when plant cpACPs were used to supplement an *E. coli* FAS system (Simoni et al., 1967), the fatty acids were apparently present as acyl-ACP intermediates and not as free fatty acids.

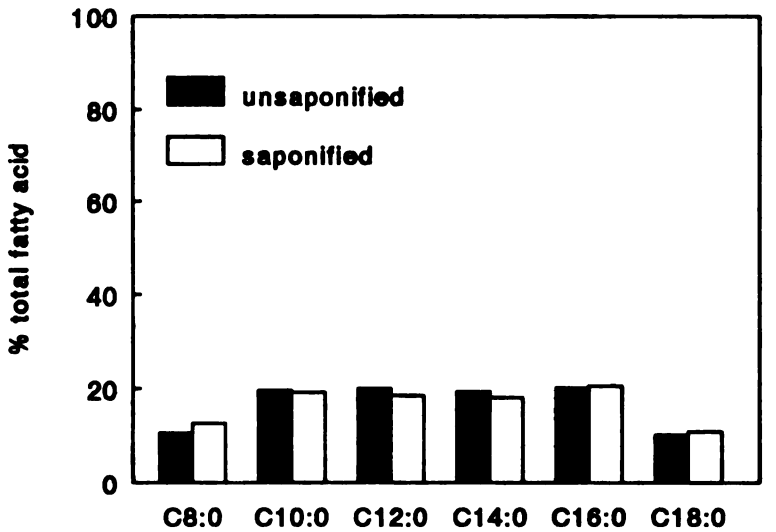
The observation of hydrolyzed short- and medium-chain fatty acids in the FAS assays supplemented with mtACP-1 was particularly surprising because the acyl-ACP thioesterases are known to be highly specific for long chain acyl-ACPs (Ohlrogge et al., 1978). It may be possible that mtACP-1 facilitates release of shorter chain fatty acids from ACP through its presentation of the acyl-ACPs to acyl-ACP thioesterases. Brody and Mikolajczyk (1988) reported that 3-hydroxytetradecanoyl-ACPs have been isolated from *Neurospora*

Figure 3-8 Product analysis of fatty acid synthase reactions supplemented with mtACP-1 or spinach cpACP-1. ^{14}C labelled fatty acids derived from unsaponifed and saponified FAS reactions supplemented with either 22.5 μM mtACP-1 or 22.5 μM spinach cpACP-1 were analyzed by reverse phase TLC and quantitated by phosphorimaging. The FAME profiles derived from unsaponified (solid bars) and saponified (open bars) FAS reactions supplemented with spinach cpACP-1 are shown in figure 8A. The FAME profiles derived from unsaponified (solid bars) and saponified (open bars) FAS reactions supplemented with mtACP-1 are shown in figure 8B. In this experiment, the total ^{14}C incorporation into the FAMEs derived from the unsaponified and saponified FAS reactions supplemented with mtACP-1 was 24,624 DPM and 28,543 DPM respectively. Unsaponified and saponified FAS reactions supplemented with cpACP-1 had total ^{14}C incorporations of 48,906 DPM and 43,746 DPM. 1 mL FAS reactions were incubated for 1 hour. Similar results were obtained in additional experiments.

A.


100
80
60
40
20
0
% total fatty acid
unsaponified
saponified
C8:0
C10:0
C12:0
C14:0
C16:0
C18:0

B.


100
80
60
40
20
0
% total fatty acid
unsaponified
saponified
C8:0
C10:0
C12:0
C14:0
C16:0
C18:0

mitochondria, suggesting that medium-chain acyl-ACP intermediates do occur on mtACPs. Furthermore, *in vivo* and *in vitro* labeling of *Neurospora* mitochondria with [2-^{14}C]-malonic acid has resulted in the synthesis of radiolabelled short- and medium-chain acyl-ACPs and free mryistic acid (Mikolajczyk and Brody, 1990). However, additional in vivo labeling experiments in Neurospora (Zenson et al., 1992) indicated that long-chain acyl intermediates were synthesizedon mtACP. Acyl-ACPs isolated from bovine mitochondria were reported to have an acyl moiety with a molecular mass of 302 D, again suggesting that a long-chain acyl intermediate could be synthesized on mtACPs (Runswick et al., 1991). Because of these divergent results, it is difficult to ascertain the significance of the accumulation of short- and medium-chain fatty acids in the chloroplast FAS assays supplemented with mtACP-1.

From the *in vitro* chloroplast FAS experiments it was apparent that mtACP-1 was structurally close enough to cpACP to at least initiate *de novo* fatty acid synthesis, but sufficiently dissimilar enough to participate differently in the fatty acid termination reactions. Although the results of these experiments could not predict what role if any mtACP-1 plays in mitochondrial *de novo* FAS, they do suggest that mtACP-1 is capable of acting as a co-factor in a FAS system. Further experiments are necessary to determine if mtACP-1 does participate in mitochondrial *de novo* FAS.

Conclusion

The cumulative results presented in this paper strongly support the conclusion that mtACP-1 represents a mitochondrial localized ACP isoform. First, mtACP-1 has been shown to be more closely related to the *Neurospora* and bovine mtACPs than to the *Arabidopsis* cpACPs. Secondly, the protein

import studies confirmed the specificity of the mtACP-1 presequence for import into mitochondria. And finally, the detection of mtACP-1 by western blot analysis in mitochondrial extracts and the apparent membrane association of the acylated form of mtACP-1 are both consistent with characteristics reported for the *Neurospora* and bovine mtACPs (Sackmann et al., 1991; Runswick et al., 1991).

Although the function of mtACP-1 has not been determined, mtACP-1 is clearly effective as a FAS co-factor in *in vitro* chloroplast FAS studies. However, further studies using isolated mitochondria will be required to determine if mtACP-1 plays a similar role in the mitochondria. Also, because of the high degree of structural similarity that mtACP-1 shares with other mtACPs, it is possible that the plant mitochondria ACPs may also be associated with NADH:ubiquinone oxidoreductase. The apparent membrane localization of acyl mtACP-1 is consistent with the association of the *Neurospora* and bovine mtACPs with the NADH:ubiquinone oxidoreductase membrane complex (Sackmann et al., 1991; Runswick et al., 1991). Regardless of the outcome, further characterization of the function of mtACP-1 will expand present ideas of the roles ACPs play in plant metabolism.

Acknowledgments

We wish to thank John Froehlich, Christie Howard, Dusty Post-Beittenmiller, and Kathy Schmid for their helpful discussion of this manuscript. We would also like to acknowledge Edgar Cahoon for help with fatty acid analysis and Linda Savage for help with chloroplast protein import studies.

Literature Cited

Battey JF, Ohlrogge JB (1990) Evolutionary and tissue-specific control of expression of multiple acyl carrier protein isoforms in plants and bacteria. Planta **180:** 352-360.

Brody S, Mikolajczyk S (1988) *Neurospora* mitochondria contain an acyl carrier protein. Eur J Biochem **173:** 353-359.

Brody S, Mikolajczyk S, Chuman L (1990) Studies on *de novo* fatty acid synthesis in mitochondria. *In* PJ Quinn, JL Harwood, eds, Plant lipid Biochemistry, Structure and Utilization. Portland Press Ltd, London, pp 117-119.

Chuman L, Brody S (1989) Acyl carrier protein is present in the mitochondria of plants and eucaryotic micro-organisms. Eur J Biochem **184:** 643-649.

Day DA, Neuburger M, Douce R (1985) Biochemical characterization of chlorophyll-free mitochondria from pea leaves. Aust J Plant Physiol, **12:** 219-228.

Elhussein SA, Miernyk JA, Ohlrogge JB (1988) Plant holo-(acyl carrier protein) synthase. Biochem J **252:** 39-45.

Fang TK, David NR, Miernyk JA, Randall DD (1987) Isolation and purification of functional pea leaf mitochondria free of chlorophyll contamination. Curr Top Plant Biochem Physiol **6:** 175.

Fernandez MD, Lamppa GK (1990a) Acyl carrier protein (ACP) import into chloroplast does not require the phosphopantetheine: evidence for a chloroplast holo-ACP synthase The Plant Cell **2:** 195-206.

Fernandez MD, Lamppa GK (1990b) Acyl carrier protein import into chloroplast: Both the precursor and mature forms are substrates for phosphopantetheine attachment by a soluble chloroplast holo-acyl carrier protein synthase. J Biol Chem **246:** 7220-7226.

Guerra DJ, Ohlrogge JB, Frentzen M (1986) Activity of acyl carrier protein isoforms in reactions of plant fatty acid metabolism. Plant Physiol. **82:** 448-453.

Hansen L (1987) Three cDNA clones for barley leaf acyl carrier proteins I and III. Carlsberg Res Commun **52:** 381-392.

Heidecker G, Messing J (1986) Structural analysis of plant genes. In WR Briggs, RL Jones, V Walbot eds, Annual Reviews of Plant Physiology, Vol 37. Annual Reviews Inc, Palo Alto, CA pp 439-466.

Hloušek-Radojčić A, Post-Beittenmiller D, Ohlrogge JB (1992) Expression of constitutive and tissue specific acyl carrier protein isoforms in *Arabidopsis.* Plant Physiol **98:** 206-214.

Issartel JP, Koronakis V, Hughes C (1991) Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. Nature **351:** 759-761.

Jackowski S, Rock CO (1987) Altered molecular form of acyl carrier protein associated with β-ketoacyl-acyl carrier protein synthase II (*fabF*) mutants. J Bacteriol **169:** 1469-1473.

Kuo TM, Ohlrogge JB (1984) Acylation of plant acyl carrier proteins by acyl carrier protein synthetase from *Escherichia coli*. Arch Biochem Biophys **230:** 110-116.

Lakin-Thomas PL, Brody S (1985) A pantothenate derivative is covalently bound to mitochondrial proteins in *Neurospora crassa*. Eur J Biochem **146:** 141-147.

Lamppa G, Jacks C (1991) Analysis of two linked genes coding for the acyl carrier protein (ACP) from *Arabidopsis thaliana* (Columbia). Plant Mol Biol **16:** 469-474.

Lin C, Thomashow MF (1992) DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor*15 and characterization of the COR15 polypeptide. Plant Physiol **99:** 519-525.

Mikolajczyk S, Brody S (1990) *De novo* fatty acid synthesis mediated by acyl carrier protein in *Neurospora crassa* mitochondria. Eur J Biochem **187:** 431-437.

Mullis KB, Faloona FA (1987) Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. Meth Enzymol **155:** 335-350.

Neurburger M, Journet EP, Bligny R, Carde JP, Douce R (1982) Purification of plant mitochondria by isopycnic centrifugation in density gradients of percoll. Arch Biochem Biophys **217:** 312-323.

Ohlrogge JB (1987) Biochemistry of plant acyl carrier proteins. In PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 10. Academic Press, New York, pp 137-157.

Ohlrogge JB, Kuo TM (1985) Plants have isoforms for acyl carrier protein that are expressed differently in different tissues. J Biol Chem **260:** 8032-8037.

Ohlrogge JB, Shine WE, Stumpf PK (1978) Fat metabolism in higher plants. Characterization of plant acyl-ACP and acyl-CoA hydrolases. Arch Biochem Biophys **189:** 382-391.

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1990) *In vivo* pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem **266:** 1858-1865.

Post-Beittenmiller MA, Hlousek-Radojcic A, Ohlrogge JB (1989) DNA sequence of a genomic clone encoding an *Arabidopsis* acyl carrier protein (ACP). Nucleic Acids Res **17:** 1777.

Rock CO, Cronan JR (1980) Improved purification of acyl carrier protein. Anal Biochem **102:** 362-364.

Roughan G (1987) Fatty acid synthesis and utilization by isolated chloroplasts. In SP Colowick, NO Kaplan, eds, Methods in Enzymology, Vol 148. Academic Press, San Diego, CA, pp 327-337.

Runswick MJ, Fearnley IM, Skehel JM, Walker JE (1991) Presence of an acyl carrier protein in NADH:ubiquinone oxidoreductase from bovine heart mitochondria. FEBS Lett. **286:** 121-124.

Sackmann U, Zensen R, Rohlen D, Jahnke U, Weiss H (1991) The acyl carrier protein in *Neurospora crassa* mitochondria is a subunit of NADH:ubiquinone reductase (complex I) Eur J Biochem **200:** 463-469.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Pro Natl Acad Sci USA **74:** 5463-5467.

Schmid KM, Ohlrogge JB (1990) A root acyl carrier protein-II from spinach is also expressed in leaves and seeds. Plant Mol Biol **15:** 765-778.

Shen B, Summers RG, Gramajo H, Bibb MJ, Hutchinson CR (1992) Purification and characterization of the acyl carrier protein of the *Streptomyces glaucescens* tetracenomycin C polyketide synthase. J Bacteriol **174:** 3818-3821.

Short JM, Fernandez JM, Sorge JA, Huse WD (1988) λ Zap: a bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Research **16:** 7583-7600.

Simoni RD, Criddle RS, Stumpf PK (1967) Fat metabolism in higher plants. XXXI. Purification and properties of plant and bacterial acyl carrier proteins. J Biol Chem **242:** 573-581.

Studier FW, Rosenburg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. In Methods of Enzymology, Vol 185. Academic Press, San Diego, CA pp 60-89.

Therisod H, Weissborn AC, Kennedy EP (1986) An essential function for acyl carrier protein in the biosynthesis of membrane-derived oligosaccharides of *Escherichia coli*. Proc Natl Acad Sci USA **83:** 7236-7240.

von Heijne G, Steppuhn J, Herrmann RG (1989) Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem **180:** 535-545.

Watanabe K, Ishikawa T, Mukohara Y, Nakamura H (1992) Cloning and sequencing of the genes involved in the conversion of 5-subsituted hydantoins to the corresponding L-amino acids from the native plasmid of Pseudomonas sp. strain NS671. J. Bacteriol. **174:** 962-969.

Wakil SJ, Stoops JK, Joshi VC (1983) Fatty acid synthesis and its regulation. Ann Rev Biochem **52:** 537-579.

Watts FZ, Walters AJ, Moore AL (1992) Characterization of PHSP1, a cDNA encoding a mitochondrial HSP70 from *Pisum sativum*. Plant Mol Biol **18:** 23-32.

Zensen R, Husmann H, Schneider R, Peine T, Weiss H (1992) *De novo* synthesis and desaturation of fatty acids at the mitochondrial acyl carrier protein, a subunit of NADH:ubiquinone oxidoreductase in *Neurospora crassa*. FEBS Lett **310:** 179-181.

Chapter 4

Feedback Inhibition of Fatty Acid Synthesis in Tobacco Suspension Cells[1].

Abstract

The addition of exogenous oleic acid, in the form of oleoyl-Tween (Tween-18:1) caused a 3 - 5 fold decrease in the rate of [1-^{14}C] acetate incorporation into tobacco suspension cell fatty acids. The decrease in acetate incorporation occurred rapidly upon addition of Tween-18:1 and appeared to be specific for fatty acid synthesis. In order to elucidate of possible regulatory steps involved in the feedback regulation of fatty acid synthesis in plant cells, tobacco cell acyl-ACP intermediates were analyzed using a combination of [1-^{14}C] acetate labelling and immuno-blot analysis. Within 30 minutes of exogenous lipid addition, acetyl-ACP increased and long chain acyl-ACP decreased, whereas medium chain acyl-ACP levels remained constant. These acyl-ACP profiles observed during the feedback inhibition were those predicted to occur under conditions where the flux through fatty acid synthesis is decreased due to limiting levels of malonyl-CoA and therefore indicated that acetyl-CoA carboxylase (ACCase) was centrally involved in the feedback regulation of fatty acid synthesis. Immuno-blot analysis showed that ACCase protein levels did not change during the feedback inhibition, suggesting that the feedback inhibition of fatty acid synthesis in plant cells may occur through biochemical or post-translational modification of ACCase and possibly other fatty acid synthesis related enzymes.

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[1]The material in this chapter was published previously [Shintani DK, Ohlrogge JB (1995) The Plant Journal 7: 577-587.

Introduction

Although the biochemistry of plant fatty acid synthesis has been extensively studied, comparatively little is known about the regulation of this pathway (Ohlrogge et al.,1993). It appears that plant fatty acid synthesis is regulated, at least in part, by the demand for fatty acids by microsomal and plastidial enzymes involved in glycerolipid synthesis. This hypothesis is supported by the observations that 1) non-esterified fatty acids do not accumulate in plant cells; 2) the lipid to protein ratios of particular membranes are stringently maintained; and 3) high rates of fatty acid synthesis are only observed in tissues where high rates of glycerolipid production are occurring such as in expanding leaves or developing oil seeds. The high degree of regulation imposed on this pathway not only helps to economize cellular energy expenditures, but also prevents the accumulation of free fatty acids and acyl-CoAs, whose detergent nature would be detrimental to the cell.

Feedback regulation of fatty acid synthesis could provide one potential mechanism that would coordinate the supply of fatty acids with this cellular demand. Feedback regulation of fatty acid synthesis has been reported in animal, bacterial, and fungal systems. The accumulation of fatty acid synthase (FAS) products, in the form of long chain acyl-CoAs, was reported to cause the inhibition of the animal (Goodridge, 1985) and yeast (Kamiryo & Numa, 1973) acetyl-CoA carboxylase (ACCase), the key regulatory enzyme of fatty acid synthesis in these organisms. Furthermore, the addition of exogenous fatty acids to bacterial cultures of *Lactobacillus* caused a reduction in de novo fatty acid synthesis (Henderson & McNeil, 1966).

The study of feedback inhibition in plants has been hindered by the inability of plants to efficiently assimilate exogenous fatty acids. However,

Terzaghi has shown that fatty acids in the form of Tween-fatty acid esters can be rapidly assimilated into the membranes of soybean suspension cells (Terzaghi, 1986a and b). When Tween-fatty acid esters were added to soybean culture medium, as much as 50% of the membrane lipids were derived from exogenous fatty acids (Terzaghi, 1986a). Labeling studies indicated that the Tween-fatty acid esters were imported into the cell where they were hydrolyzed to yield free fatty acids. When Tween-fatty acid esters of oleic or stearic acid were added to the culture medium of soybean suspension cultures, ^{14}C acetate incorporation into the fatty acids decreased relative to untreated cells (Terzaghi, 1986b). This observation provided preliminary evidence that fatty acid synthesis could be regulated by feedback inhibition in plants.

In the present study, the Tween-fatty acid feeding system was adapted for use in tobacco suspension cell cultures. The effect of exogenous fatty acids on individual acyl-acyl carrier protein (ACP) intermediates of plant fatty acid synthesis was investigated using a combination of ^{14}C-acetate labelling and immuno-blot analysis with antibodies to ACP. The information obtained from these studies has been used to deduce possible enzymatic site(s) involved in the feedback regulation of plant fatty acid synthesis. The results presented here indicated that biochemical feedback regulation of FAS is responsible for the decreased rate of acetate incorporation into fatty acids of Tween-18:1 treated tobacco suspension cells, and that acetyl-CoA carboxylase is the most likely site of action of the feedback regulation.

Materials and Methods

Cell culture and medium.

Tobacco (*Nicotiana tabacum* L. cv bright yellow 2) cells were grown

shaking at 28°C in 50 ml of liquid medium composed of Murashige and Skoog basal salts (Gibco-BRL, Grand Island, NY), 3% sucrose, 2.5 mM Mes/KOH pH 5.7, 1 mg/ml thiamine, 1 mg/ml myo-inositol, 1 μM 2,4-D. Cultures were subcultured weekly with a 5% (v/v) inoculum from a seven day old culture.

Determination of the effect of Tween-18:0 and Tween-18:1 on cell growth.

Tween-18:0 and Tween-18:1 solutions were prepared based on the determination of Terzaghi (1986) that commercial Tween 80 is approximately 20% (w/w) oleic acid. Therefore a 90 mM Tween-18:1 stock was prepared by diluting 6.4 gm of tissue culture grade Tween 80 (Sigma, St. Louis, MO) in 50 ml of water. A 90 mM Tween-18:0 stock solution was prepared by diluting 7.1 gm of Tissue culture grade Tween 60 (Sigma, St. Louis, MO) in 50 ml of water. 50 ml tobacco cell cultures were grown in Tween-18:0 and Tween-18:1 concentrations ranging from 225 μM to 900 μM and 450 μM to 900 μM respectively for 11 days. The packed cell volume was determined daily after pelleting the cells at 1000 rpm for 5 minutes. The medium was aspirated away and the cells were resuspended in fresh medium and fresh Tween-18:0 and 18:1 was added to maintain the initial concentration.

Acetate labelling.

Tobacco cells were used four days after subculturing. To maintain homogeneity between treatments, two separate 50 ml cultures were combined, mixed and then realiquoted. At various times after the addition of Tween-18:1 to a final concentration of 900 μM to one of the two cultures, 2.5 ml of cells were sampled and immediately added to a tube containing 25 μCi of [1-^{14}C] acetate (47 - 54 Ci/mole) (New England Nuclear, Boston, MA). The cells were

then incubated for 10 minutes shaking at 28°C after which the labelling was quenched by the addition of TCA to a final concentration of 5%. The TCA treated cells were then immediately frozen in liquid nitrogen and stored at -80°C. The TCA treated cells were thawed on ice and homogenized for approximately 30 seconds using a Brinkmann polytron at the highest setting. The homogenates were then centrifuged at 10,000 g for 20 minutes at 4°C. The resulting supernatant was aspirated away and the pellet was washed in 1% TCA. The washed cell pellet was then resuspended in 2 ml of 50 mM Hepes pH 7.8, 10 mM N-ethylmaleimide (NEM). Protein determination of this homogenate was done as described by Lowry et al., (1951). The homogenate was then centrifuged at 10,000 g for 20 minutes at 4°C and the resulting pellet and supernatant were saved for lipid and acyl-ACP analysis respectively.

Lipid analysis.

Total lipid was extracted from the cell pellet remaining after resuspending the TCA precipitate in 50 mM Hepes pH 7.8, 10 mM NEM by the method described by Bligh and Dyer (1959). Methyl esters were derivatized from the total lipid extract as described by Morrison and Smith (1964) using boron trichloride. The total lipid extract was dried under N_2 and resuspended in 300 µl of toluene. 1 ml of 10% (w/v) boron trichloride in methanol (Alltech) was added and the mixture was incubated at 90°C for 1 hour. The mixture was allowed to cool and 1 ml of water was added. Methyl esters were then extracted three times with hexane.

To determine the amount of [1-^{14}C] acetate incorporated into the fatty acid methyl ester (FAME) fraction, the total methyl ester mixture was fractionated on silica 60 A TLC plates (Whatmann) developed in 60:40:1 (v:v:v) hexane:diethyl

ether:acetic acid. The FAME fraction was identified by co-migration with ^{14}C labelled FAME standards and then scraped from the TLC plate. The amount of ^{14}C-label incorporated into FAMEs was determined by liquid scintillation counting.

The amount of label incorporated into individual fatty acid species was determined by first fractionating the total methyl ester fraction by argentation TLC. Argentation plates were prepared by first saturating silica 60 A TLC plates (Whatmann) with 15% $AgNO_3$ in acetonitrile and then drying them overnight. Saturated FAMEs and individual unsaturated FAMEs were fractionated in three developments of the argentation TLC plates in toluene at -20*C. Unsaturated FAME species were scraped from the TLC plates and counted by liquid scintillation counting. Saturated FAMEs were extracted three times from the argentation plate scrapings with 2:1 (v:v) hexane:diethyl ether and further fractionated on KC18 reverse phase TLC plates (Whatmann) developed in 65:35:0.5 (v:v:v) acetonitrile:methanol:water. Individual saturated FAME species were scraped from the TLC plates and counted by liquid scintillation counting.

Analysis of ^{14}C-labelled acyl-ACPs.

The supernatant derived from the resupended TCA precipitate was applied to a 0.5 ml DEAE cellulose column (Sigma) equilibrated with 10 mM Mes pH 6.1. The column was then washed with 6 column volumes of 10 mM Mes pH 6.1. The acyl-ACPs were eluted from the column with 3 column volumes of 0.5 M LiCl in 10 mM Mes pH 6.1. The eluted acyl-ACPs were concentrated by precipitation in 5% TCA. The acyl-ACPs were then resuspended in 500 µl of 50 mM Hepes pH 7.8, 10 mM NEM. The acyl-ACPs

were fractionated on a 1 M urea 15% native acrylamide gel and blotted onto nitrocellulose filters as described by Post-Beittenmiller et al. (1991). The filters were then wrapped in 1.5 μm mylar and used to expose phosphor-image screens (Molecular Dynamics) for four days. ^{14}C-labelled acyl-ACPs were identified by comparing their migration to ^{14}C labeled acyl-ACP standards synthesized *in vitro* using *E. coli* acyl-ACP synthetase (Kuo & Ohlrogge, 1984). The labeled acyl-ACPs were then cut from the nitrocellulose filters. The acyl-ACPs were derivatized to FAMEs by dissolving the nitrocellulose pieces in 1.5 ml of 0.5 M sodium methoxide in methanol and incubated at room temperature for 1 hour. The derivatization was stopped by adding 1.5 ml of 125 mM H_2SO_4 and the FAMEs were then extracted three times with 3 ml of hexane. Individual FAME species derived from the acyl-ACP fraction were fractionated and counted as described above.

Analysis of ACP-SH, acetyl-ACP, and malonyl-ACP species.

Tobacco ACPs were isolated from 50 ml of seven day old tobacco suspension cell cultures as described by Shintani and Ohlrogge (1994) up to the DEAE chromatography step. Tobacco ACPs were deacylated by incubation at 65°C for 1 hour in 100 mM Tris-HCl, pH 9.0, 200 mM DTT. Malonyl-ACP standards were prepared from the deacylated tobacco ACPs using malonyl transferase isolated from *E. coli* as described by Stapleton and Jaworski (1983). Acetyl-ACP standards were prepared chemically by reacting the deacylated tobacco ACPs with acetyl-imidazole as described by Cronan and Klages (1981). Tobacco acetyl-ACP, malonyl-ACP, and free ACP species were fractionated by 15% native PAGE as described by Post-Beittenmiller et al. (1991). The acetyl-ACP, malonyl-ACP, and free ACP fractions from control and Tween-18:1

treated tobacco cells were isolated as described above for the radiolabelled acyl-ACPs.

Immuno-blot Analysis of FAS Enzymes

5 ml aliquots were taken at various times after the addition of 900 μM Tween-18:1 to one of two 4 day old tobacco cell cultures. The cells were pelleted and frozen in liquid N_2 and stored at -70°C. The cells were thawed on ice in 50 mM Hepes pH 7.5, 1 mM PMSF, 1 mM benzamidine, 1 μg/ml leupeptin and homogenized using a Brinkmann polytron at the highest setting for 1 minute. The homogenates were clarified by centrifuging at 10,000 g for 20 minutes at 4°C. 100 μg of protein from the supernatant of each sample was run on 10% SDS PAGE gels (Alternatively, the cytosolic ACCase which was run on a 6% SDS PAGE gel). The gels were blotted onto nitrocellulose. Each filter was blocked with 5% low fat powdered milk in 10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween 20 (TBST) for 1 hour at room temperature. The cytosolic ACCase and the BCCP subunit of the plastid ACCase were identified using 1/500 dilution of commercial alkaline phosphatase conjugated antibody to biotin (Sigma, St Louis, MO). The biotin carboxylase subunit of the plastid ACCase was identified using a 1/500 dilution of an antibody against the caster bean endosperm biotin carboxylase (Keith Roesler, personal communication).

Results and Discussion

Development of Tween feeding system for tobacco suspension cells

The Tween feeding system developed by Terzaghi (1986a) was adapted for use with tobacco cell cultures. Initial studies were conducted to determine if tobacco cells would respond as soybean cells did to the addition of oleoyl-

Tween (Tween-18:1) and stearoyl-Tween (Tween-18:0). Tobacco cells were grown in the presence of various concentrations of either Tween-18:0 or Tween-18:1 to determine the maximal concentration of each that could be tolerated without effecting cell growth. In these experiments, tobacco cells were able to grow at rates essentially the same as control cells in 900 μM Tween-18:1 (Fig. 4-1b) and were later found to tolerate concentrations as high as 1800 μM (data not shown). This high tolerance to Tween-18:1 was similar to results reported for soybean cells (Terzaghi, 1986a). However, while soybean cells were able to tolerate relatively high concentrations of Tween-18:0 (i.e. 1000 μM), tobacco cell growth was inhibited when Tween-18:0 was added at concentrations as low as 225 μM (Fig. 4-1a).

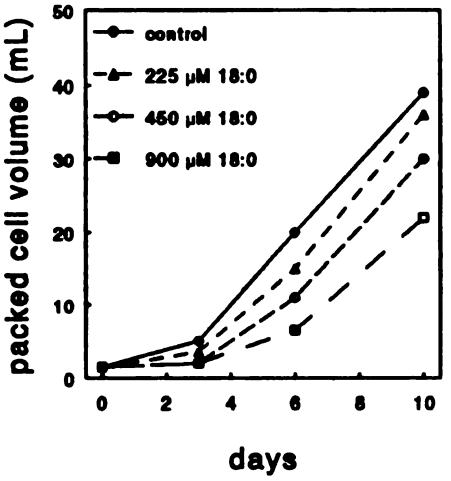
The addition of either Tween-18:0 or Tween-18:1 caused significant decreases in acetate incorporation into total lipid (Fig. 4-1c). While the addition of Tween-18:0 caused a 2 fold decrease in acetate incorporation, the Tween-18:1 treatment proved to be a more potent inhibitor of lipid synthesis, causing a 3 - 5 fold decrease in label accumulation (Fig. 4-1c). Since Tween-18:1 was less toxic to cell growth and more effective in inhibiting acetate incorporation into total lipid relative to Tween-18:0, all subsequent studies focused on the effect of Tween-18:1 on tobacco cell fatty acid synthesis.

Tween-18:1 inhibits [1-^{14}C] acetate incorporation into fatty acids.

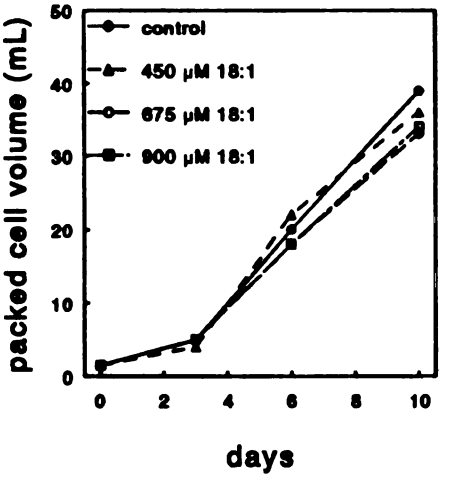
The effect of Tween-18:1 on the rate of [1-^{14}C] acetate incorporation into fatty acids was both rapid and dramatic. Exponentially growing tobacco cell cultures treated with 900 μM Tween-18:1 showed a 3 to 5 fold decrease in the rate of [1-^{14}C] acetate incorporation into fatty acids relative to control cells (Fig. 4-2a). This decrease in label accumulation was detected as early as 15 minutes

Fig. 4-1a,b,c Effect of Tween-18:0 and Tween-18:1 on tobacco cell growth and acetate incorporation into total lipid fraction. **a.** Cell growth measured in cultures treated with 0 μM (closed circle), 225 μM (open triangle), 450 μM (open circle), or 900 μM (open square) Tween-18:0 **b.** Cell growth measured in cultures treated with 0 μM (closed circle), 450 μM (open triangle), 675 μM (open circle), and 800 μM (open square) Tween-18:1. **c.** [1-^{14}C] acetate incorporation into total lipid of untreated cells (closed circle), cells treated with 225 μM Tween-18:0 (open triangle) and cells treated with 900 μM Tween-18:1 (open squares).

a.


control
225 µM 18:0
450 µM 18:0
900 µM 18:0
packed cell volume (mL)
0
10
20
30
40
50
0
2
4
6
8
10
days

b.


control
450 µM 18:1
675 µM 18:1
900 µM 18:1
packed cell volume (mL)
0
10
20
30
40
50
0
2
4
6
8
10
days

c.

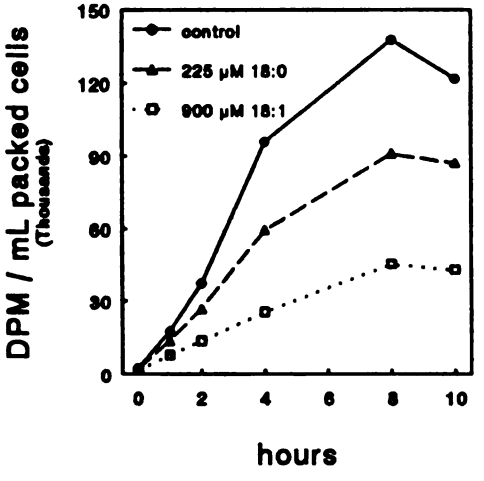
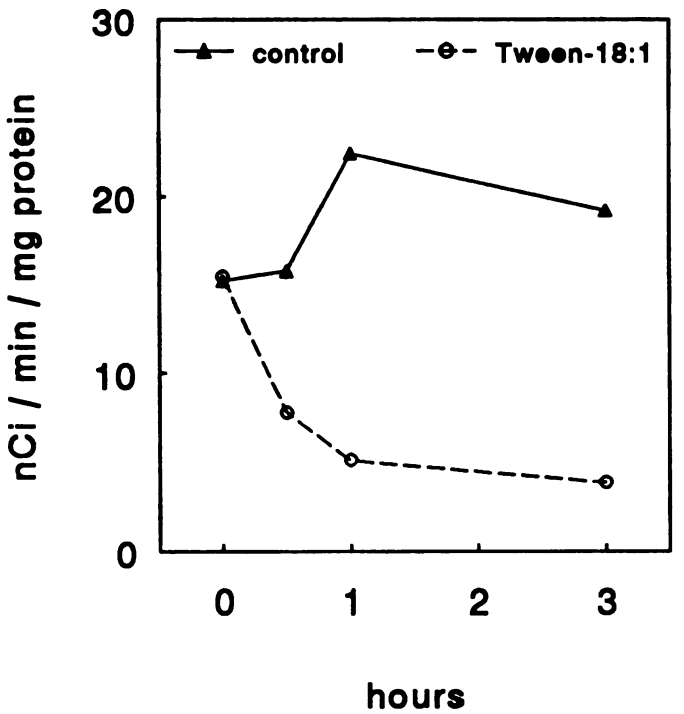
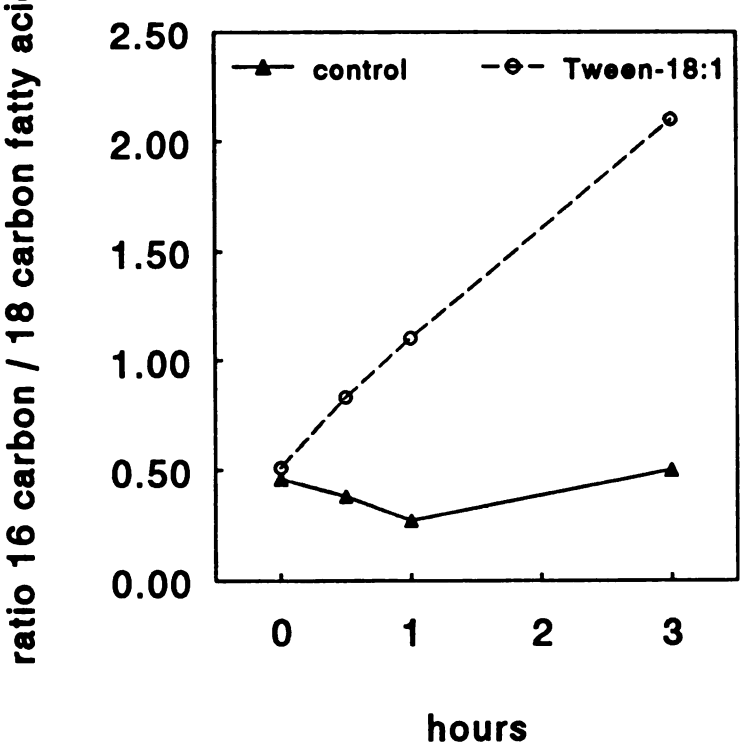

control
225 µM 18:0
900 µM 18:1
DPM / mL packed cells
(Thousands)
0
30
60
90
120
150
0
2
4
6
8
10
hours

Fig. 4-2a,b The effect of Tween-18:1 on the rate of [1-^{14}C] acetate incorporation into fatty acids of tobacco cells. a. Effect of Tween-18:1 on the rate of [1-^{14}C] acetate incorporation into the total fatty acid fraction of untreated cells (closed triangles) and cells treated with 900 μM Tween-18:1 (open circles). 2.5 ml of cells were incubated for 5 minutes in 25 μCi of [1-^{14}C] acetate. Labelling was stopped by adding TCA to 5% (v/v). The total lipid was extracted from the TCA pellet and fatty acids were derivatized with boron trichloride. Fatty acid methyl-esters (FAMEs) were purified by TLC after which [1-^{14}C] acetate incorporation was determined by scintillation counting. **b.** Ratio of *de novo* synthesized 16 carbon fatty acids to 18 carbon fatty acids in untreated cells (closed triangles) and Tween-18:1 treated cells (open circles). Individual FAMEs species were fractionated first by argentation TLC and then by reverse phase TLC. Each FAME species was scraped from the TLC plates and [1-^{14}C] acetate incorporation was determined by scintillation counting.

a.


control
Tween-18:1
nCi / min / mg protein
30
20
10
0
0
1
2
3
hours

b.


control
Tween-18:1
ratio 16 carbon / 18 carbon fatty acids
2.50
2.00
1.50
1.00
0.50
0.00
0
1
2
3
hours

after Tween-18:1 addition (data not shown) and continued to decrease for one hour (Fig. 4-2a), after which a minimal rate of fatty acid synthesis was maintained for at least eight hours. In contrast, control cells showed an increased rate of [1-^{14}C] acetate incorporation into fatty acids after 1 hour and then maintained this rate for several hours (Fig.4-2a). The increasing rate of acetate incorporation into fatty acids in control cultures was consistent with the observation that sycamore cells in log phase growth show an increase in both fatty acid and phospholipid content (De Silva & Fowler, 1976). Although *de novo* fatty acid synthesis was inhibited in Tween-18:1 treated cells, the amount of oleate derived from the exogenously supplied Tween-18:1 was sufficient to maintain membrane synthesis rates necessary for exponential growth.

Although the results of the [1-^{14}C] acetate labelling experiments suggested that the addition of an exogenous supply of lipid can lead to the feedback inhibition of fatty acid synthesis, care was taken when interpreting these results because the use of [1-^{14}C] acetate in fatty acid labelling experiments could have lead to erroneous conclusions due to the alteration of endogenous acetate pools (Nunn et al., 1977). Several lines of evidence were examined to confirm that the decrease in acetate incorporation into the fatty acids of Tween-18:1 treated cells was due to the inhibition of the fatty acid synthesis and not due to alterations in the endogenous acetate pool. First, the decrease in acetate incorporation into the fatty acids of Tween-18:1 treated cells was observed over a 25 fold range of [1-^{14}C] acetate concentrations (i.e. 40 μM to 1 mM) (data not shown). Since the Tween-18:1 induced inhibition of acetate incorporation was seen over a large range of [1-^{14}C] acetate concentrations, it was unlikely that the inhibition was due to an alteration in endogenous acetate pool sizes. Secondly, the Tween-18:1 treatment did not inhibit [1-^{14}C] acetate incorporation into sterols, indicating that

the inhibitory effect of Tween-18:1 was specific to fatty acid synthesis. In these experiments, the rate of [1-^{14}C] acetate incorporation into sterols was 0.52 μCi/gm fresh weight/minute for Tween-18:1 treated cells versus 0.43 μCi/gm fresh weight/minute for control cells. If the Tween-18:1 treatment altered endogenous acetate pools then the rate of radiolabel incorporated into sterols would also be expected to have been reduced. Finally, as will be discussed later, the Tween-18:1 induced decrease in radiolabel accumulation into fatty acids was accompanied by an alteration in the profile of several acyl-ACP intermediates that was consistent with decreased fatty acid synthesis. These cumulative findings indicated that the addition of Tween-18:1 to tobacco cultures was inhibiting acetate incorporation into fatty acids as the result of the feedback regulation of fatty acid synthesis.

Tween-18:1 alters the products of fatty acid synthesis.

In addition to its inhibitory effect on acetate incorporation, Tween-18:1 also had an effect on fatty acid chain length and desaturation. Compared to control cells, the Tween-18:1 treated cells accumulated significantly less label into the total 18 carbon fatty acid fraction (i.e. stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3)) relative to the amount incorporated into palmitic acid (C16:0) (Fig. 2b). The addition of Tween-18:1 resulted in a four to five fold increase in the ratio of labelled 16 carbon fatty acids relative to labelled 18 carbon fatty acids, whereas this ratio remained constant in control cells (Fig. 4-2b). Because linolenic acid was the major fatty acid found in the membranes of tobacco suspension cells (data not shown), the decrease in 18 carbon fatty acid synthesis in Tween-18:1 treated cells also had a major effect on the degree of fatty acid desaturation. Similar results were

reported for soybean cells where the ratio of saturated to unsaturated fatty acids was found to increase as a result of Tween-18:1 feedings (Terzaghi, 1986b).

The results shown in Fig. 2b suggest that Tween-18:1 treated tobacco cells were able to detect changes in the degree of membrane fatty acid desaturation and then respond by modulating not only the quantity, but also the quality of *de novo* synthesized fatty acids. Although the total rate of fatty acid synthesis was decreased in Tween-18:1 treated cells, the remaining FAS activity was redirected toward the production of predominantly palmitic acid. The majority of the oleate, linoleate and linolenate of treated cells were derived from the exogenously supplied Tween-18:1, and therefore the cells were not required to synthesize oleate via *de novo* fatty acid synthesis. Presumably the cells adapt the products of *de novo* fatty acid synthesis to maintain some level of saturated fatty acid synthesis while attenuating the level of unsaturated fatty acid synthesis in Tween-18:1 treated cells. We considered that the Tween 18:1 treatment caused a decrease in the activity of the enzyme that catalyzes the elongation of 16 carbon fatty acids to 18 carbon fatty acids, β-keto acyl-ACP synthase II (KAS II). However, as will be discussed later, the acyl-ACP profile of the Tween-18:1 treated cells was inconsistent with this possibility.

Analysis of tobacco cell acyl-ACP intermediates.

The steady state pool sizes of the acyl-ACP intermediates of fatty acid synthesis reflect the balance between their rate of synthesis and rate of turnover (Soll & Roughan, 1988; Jaworski et al., 1993). Alterations in these pools in response to changes in the rates of fatty acid synthesis have been used to identify acetyl-CoA carboxylase (ACCase) as a regulatory enzyme involved in light/dark control of fatty acid synthesis (Post-Beittenmiller et al., 1991, 1992b). In the

present study, the relative pool sizes of several acyl-ACP intermediates were examined in control and Tween-18:1 treated cells in order to deduce possible site(s) of regulation involved in the feedback inhibition of fatty acid synthesis.

Tween-18:1 alters [1-^{14}C] acetate incorporation into long chain acyl-ACPs.

The addition of Tween-18:1 caused significant changes in the amount of [1-^{14}C] acetate incorporated into the C16:0 and C18:0 acyl-ACP pools. While the amount of label associated with the C16:0 and C18:0 acyl-ACP pools in control cells increased over time, treated cells responded rapidly to the addition of Tween-18:1 by incorporating less acetate into these acyl-ACP pools (Fig. 4-3a and 4-3b). The decrease in acetate incorporation into the C16:0 and C18:0 acyl-ACP pools was detected as early as 30 minutes after the addition of Tween-18:1. The profile of [1-^{14}C] acetate incorporation into the C16:0 and C18:0 acyl-ACP pools (Fig 4-3a and 4-3b) of both the control and treated cells closely coincided with the profile of acetate incorporation into the fatty acid fractions of these cells (Fig. 4-2a). No label was detected in the oleoyl-ACP fraction of either the Tween-18:1 treated or control cells (data not shown). This result was consistent with the observation that plant extracts contain very active long chain acyl-ACP thioesterases that show a substrate preference for oleyl-ACP (Ohlrogge et al., 1978).

Since the synthesis of the C16:0 and C18:0 acyl-ACPs was dependent on condensation of a malonyl-ACP molecule with an acyl-ACP, the decreased formation of these acyl-ACPs in Tween-18:1 treated cells was consistent with either a decrease in available malonyl-ACP or the decrease in the rate of the condensation reaction catalyzed by KAS II. Since an accumulation of 16:0-ACP and medium chain acyl-ACPs was not observed in Tween-18:1 treated cells (Fig

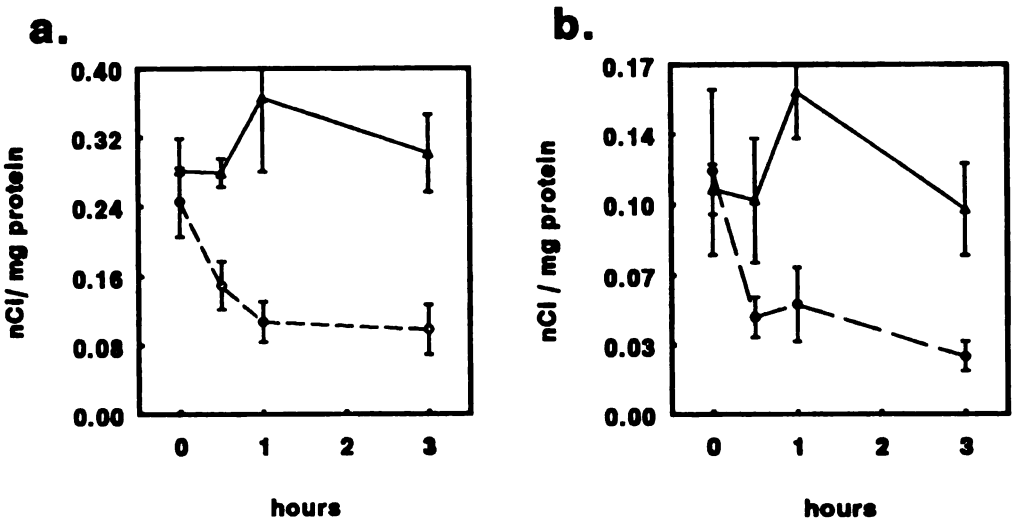


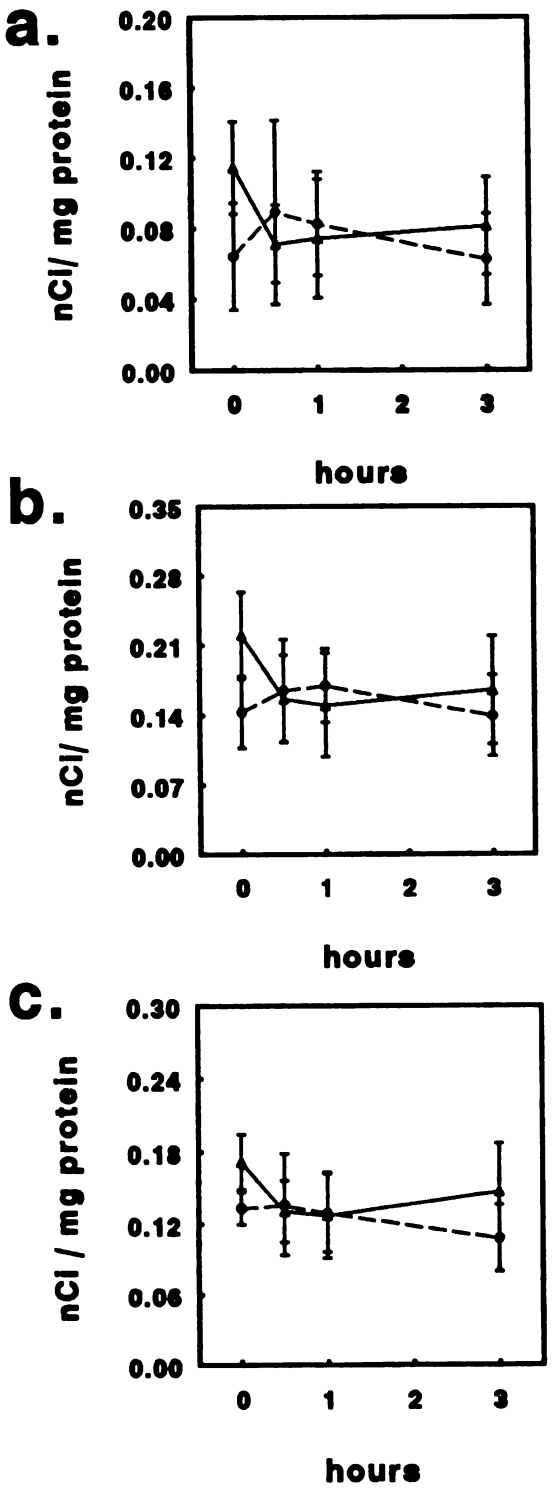
Fig. 4-3a,b. Effect of Tween-18:1 on [1-^{14}C] acetate incorporation into long chain acyl-ACPs of tobacco cells. a. [1-^{14}C] acetate incorporated into 16:0-ACP fraction of untreated cells (closed triangles) and cells treated with 900 μM Tween-18:1 (open circles). **b.** [1-^{14}C] acetate incorporated into 18:0-ACP fraction of untreated cells (closed triangles) and cells treated with 900 μM Tween-18:1 (open circles). All values were derived from the mean of three individual experiments. Error bars represent standard error.

4-4a,b,c), it was unlikely that the decrease in C16:0 and C18:0 acyl-ACPs pools was the result of inhibition occurring at KAS II. It was more likely that the decrease in long chain acyl-ACP synthesis in Tween-18:1 treated cells was due to a decrease in available malonyl-ACP caused by the inhibition of ACCase.

The reduced [1-^{14}C] acetate incorporation and the depletion of the long chain acyl-ACP pools in Tween-18:1 treated plants indicated that although the flux through fatty acid synthesis was reduced by the addition of Tween-18:1, this treatment did not substantially inhibit the turnover of these acyl-ACP pools. The turnover of the C16:0 and C18:0 acyl-ACP pools is due to the presence of very active long chain acyl-ACP thioesterases. These results suggest that the activity of these thioesterases is not coupled to the feedback regulation of fatty acid synthesis in tobacco cells.

The long chain acyl-ACP pools from developing seeds and light/dark exposed chloroplasts responded similarly to changes in the rates of fatty acid synthesis as did the feedback inhibited tobacco cells. Early in seed development, when very active fatty acid synthesis is occurring, long chain acyl-ACPs have been reported to accumulate to relatively high levels (Post-Beittenmiller et al., 1992a). However, later as the rate of fatty acid synthesis decreased, the long chain acyl-ACP pools became depleted (Post-Beittenmiller et al., 1992a). Similarly, in spinach chloroplasts, long chain acyl-ACP levels remained high in the light when rates of fatty acid synthesis were high and decreased in the dark when fatty acid synthesis was inhibited (Soll and Roughan, 1982; Roughan and Nishida, 1990). These observations suggested that long chain acyl-ACPs were probably not directly involved in the perception of the feedback inhibition because these acyl-ACPs were reduced during the inhibition of fatty acid synthesis. However, long chain acyl-ACPs have recently been

Fig. 4-4a,b,c. Effect of Tween-18:1 on [1-^{14}C] acetate incorporation into medium chain acyl-ACPs of tobacco cells. **a.** [1-^{14}C] acetate incorporated into-10:0-ACP fraction of untreated cells (closed triangles) and cells treated with 900 μM Tween-18:1 (open circles). **b.** [1-^{14}C] acetate incorporated into 12:0-ACP fraction of untreated cells (closed triangles) and cells treated with 900 μM Tween-18:1 (open circles). **c.** [1-^{14}C] acetate incorporated into 14:0-ACP fraction of untreated cells (closed triangles) and cells treated with 900 μM Tween-18:1 (open circles). All values were derived from the mean of three individual experiments. Error bars represent standard error.


a.
0.20
0.16
0.12
0.08
0.04
0.00
nCl/ mg protein
0
1
2
3
hours
b.
0.35
0.28
0.21
0.14
0.07
0.00
nCl/ mg protein
0
1
2
3
hours
c.
0.30
0.24
0.18
0.12
0.06
0.00
nCl / mg protein
0
1
2
3
hours

suggested as a potential feedback inhibitor of bacterial fatty acid synthesis (Jiang and Cronan, 1994; Ohlrogge et al., manuscript submitted) and it is possible that under other conditions this molecule may participate in the regulation of plant fatty acid synthesis.

Medium chain acyl-ACP pools are not altered by Tween-18:1 treatment.

While the addition of Tween-18:1 caused a decrease in acetate incorporation into the long chain acyl-ACP pools, this treatment had no effect on the medium chain acyl-ACPs. The amount of [1-^{14}C] acetate incorporated into the C10:0, C12:0, and C14:0 acyl-ACP pools did not change for at least 3 hours in both Tween treated and control cells (Fig. 4-4a - 4-4c).

These results indicated that although the total flux through the fatty acid synthetic pathway decreased upon the addition of Tween-18:1, the relationship between the rate of synthesis and the rate of turnover of the medium chain acyl-ACPs remained unchanged. Because both the synthesis and turnover of the medium chain acyl-ACP intermediates is dependent on the condensation of the elongating fatty acid chain with a malonyl-ACP molecule, the constant level of medium chain acyl-ACP intermediates could be explained by either a decrease in available malonyl-ACP or by inhibition occurring at the enzyme responsible for the condensation reaction, β-keto acyl ACP synthase I (KAS I). If KAS I were being inhibited in response to the addition of Tween-18:1, an increase in the sizes of the short chain (i.e. 4 through 8 carbon) acyl-ACP and malonyl-ACP pools should have been observed. Such an acyl-ACP profile was seen in spinach chloroplast extracts treated with cerulinin, a potent inhibitor of KAS I (Jaworski et al., 1993). No such increase was detected in shorter chain acyl-ACP pools of the Tween-18:1 treated tobacco cells. The simplest interpretation consistent

with the observation that the sizes of the medium chain acyl-ACP pools remained constant in the presence and absence of Tween-18:1 is that the decreased flux through the fatty acid synthesis pathway was due to a decrease in malonyl-ACP corresponding to a decrease in malonyl-CoA as a result of the feedback inhibition of ACCase.

The results in figure 4-4 also indicated that the decreased rate of fatty acid synthesis was not due to inhibition occurring solely at the initial condensation reaction catalyzed by β keto-acyl-ACP synthase III (KAS III). If inhibition of fatty acid synthesis was occurring at KAS III, one would expect that the malonyl-ACP that was no longer being used by KAS III, would be utilized in other condensation reactions such as those catalyzed by KAS I. If no newly initiated acyl-ACP precursors were synthesized, then the medium chain acyl-ACPs would be rapidly depleted as they were elongated. Because the medium chain acyl-ACP pools were not depleted upon the addition of Tween-18:1, it was not likely that the inhibition of KAS III was solely responsible for the decreased rate of fatty acid synthesis.

Tween-18:1 altered the sizes of the acetyl-ACP and free ACP pools.

The tobacco cell acetyl-ACP, malonyl-ACP and free ACP-SH pools were analyzed by native PAGE immuno-blot analysis as described by Post-Beittenmiller et al. (1991). Because tobacco cells contained two major ACP isoforms, it was first necessary to identify individual free, acetyl- and malonyl-ACP species in cell extracts by their co-migration with *in vitro* prepared standards. In the case of both isoforms, the malonyl-ACP and acetyl-ACP species were clearly resolved from the free ACP species (Fig. 4-5a).

Immuno-blot analysis indicated that the addition of Tween-18:1 caused

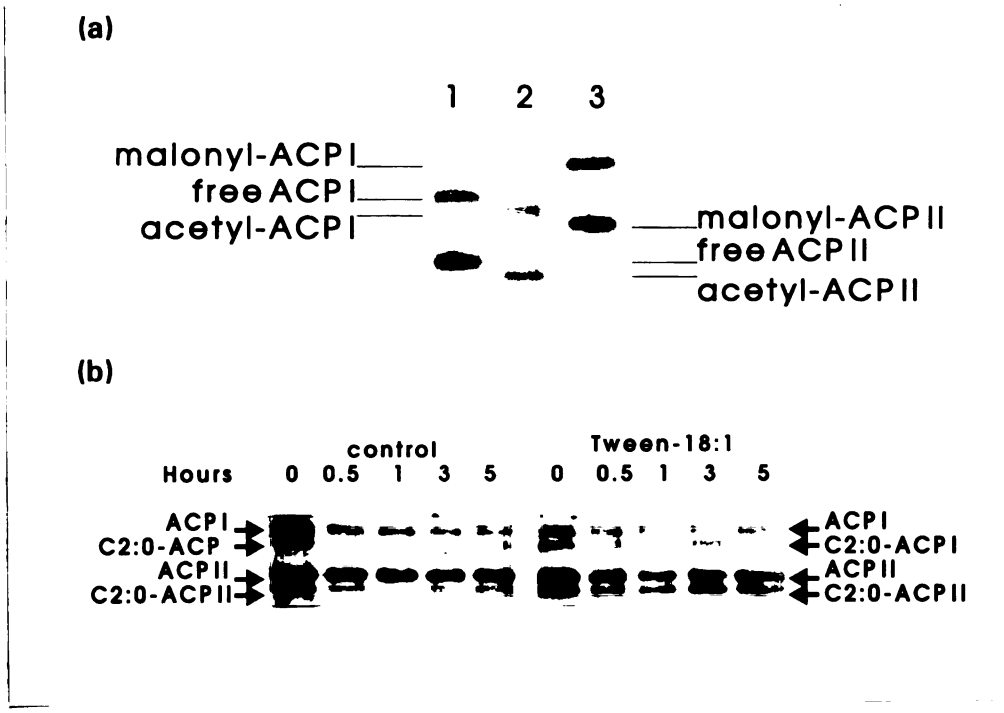


Fig. 4-5a, b. Analysis of the free-, acetyl-, and malonyl-ACPs pools from control and Tween-18:1 treated tobacco cells. **a)** ACP standards were prepared *in vitro* from deacylated ACPs isolated from tobacco cells. Lane 1, free ACP standards; lane 2, acetyl-ACP standards; and lane 3, malonyl-ACP standards. **b)** Acyl-ACPs were extracted using 5% TCA from treated and untreated cells at various times after the addition of Tween-18:1. TCA precipitated proteins were resuspended in 50 mM HEPES pH 7.8, 10 mM NEM. Approximately 100 μg of TCA precipitated protein was loaded onto each gel. All samples were run on 15% native PAGE gels and blotted to nitrocellulose. ACP species were then identified using antibodies made to spinach ACP.

changes in the free and acetyl-ACP pools. As the rate of [1-^{14}C] acetate incorporation into fatty acids decreased in Tween-18:1 treated cells (Fig. 4-2a), a coincident decrease in free ACP level was observed (Fig. 4-5b). In control cells where fatty acid synthesis was not inhibited (Fig. 4-2a), the free ACP levels appeared to remain constant over the course of these experiments (Fig. 4-5b).

The acetyl-ACP levels had an inverse relationship to the rates of [1-^{14}C] acetate incorporation into the fatty acids of tobacco cells. The acetyl-ACP levels increased after the addition of Tween-18:1 (Fig. 4-5b) and continued to increase to a maximum level at 3 hours which coincided with the minimum rate of acetate incorporation into the total fatty acid fraction (Fig. 4-2a). This increase of acetyl-ACP levels was apparent within 30 minutes after the addition of Tween-18:1. Conversely, in control cells where label incorporation into fatty acids was not inhibited (Fig. 4-2a), acetyl-ACP levels remained low (Fig. 4-5b). However, as the rate of fatty acid synthesis began to plateau between 1 and 3 hours (Fig. 4-2a), a slight increase in acetyl-ACP levels in the control cells could be detected (Fig. 4-5b).

Although the free ACPs and acetyl-ACPs were easily resolved by immuno-blot analysis, malonyl-ACP levels were below the level of detection. Several attempts to increase the sensitivity of the analysis, including specific radiolabelling of the malonyl-ACPs with ^{14}C bicarbonate, failed, suggesting that either cellular levels of malonyl-ACP were extremely low or that the tobacco cell malonyl-ACP was particularly labile. The former appeared to be more likely since *in vitro* synthesized tobacco cell malonyl-ACP standard proved to be stable during electrophoresis and could be stored at -20°C for several months with no detectable breakdown.

The changes observed in the free and acetyl-ACP pools of Tween-18:1

treated tobacco cells are similar to free and acetyl-ACP pools of spinach chloroplast during dark inhibition of FAS where it was concluded that ACCase was being down regulated (Post-Beittenmiller et al., 1991, 1992b). In both spinach leaf and tobacco suspension cultures, as the rate of FAS decreased, the levels of acetyl-ACPs increased and the levels of free ACPs decreased. Jaworski et al. (1993) explained that the increase in acetyl-ACP was in response to the inhibition of malonyl-CoA synthesis by ACCase and the uninhibited acetyl-transferase activity of enzyme β keto-acyl-ACP synthase III (KAS III).

The feedback regulation of fatty acid synthesis was not caused by the attenuation of ACCase levels.

Even though the acyl-ACP analysis implicated ACCase as an important regulatory enzyme in fatty acid synthesis, the mechanism by which the regulation occurred could not be deduced from those experiments. If the feedback control mechanisms regulating fatty acid synthesis in plant cells were similar to yeast and animals, then two different modes of regulation could have been responsible for the feedback inhibition of fatty acid synthesis. First, in yeast and animals, "short term" regulation of fatty acid synthesis has been shown to occur through allosteric and covalent modifications of ACCase (Kim, 1983; Nikawa et al., 1979). This type of regulation provides stringent control of fatty acid synthesis and allows the organism to rapidly respond to momentary changes in cellular demands for fatty acid synthesis. In addition to this "short term" control, animals, yeast and bacteria response to chronic changes in the cellular demand for fatty acid synthesis by altering the levels of ACCase protein and other FAS related enzymes. When exogenous fatty acids were added to the culture medium of yeast, rat hepatocytes, and human skin fibroblast, a significant decrease in the

levels of ACCase was observed (Kamiryo and Numa, 1973; Kitajima et al., 1975; Jacobs et al., 1973). Additionally, Li and Cronan (1993) have reported that levels of *E. coli* ACCase directly correlated with the rate of cell growth. The activity of other fatty acid synthetic enzymes have also been shown to be regulated at the level of gene expression. For example, when rats were fed a diet rich in polyunsaturated fats, the levels of FAS mRNA decreased (Clarke et al., 1990). In *Lactobacillus plantarum,* the levels of ACP were reduced to 80% of control levels when these bacteria were fed exogenous fatty acid (Sabaitis JE and Powell GL, 1976). Furthermore, the level of yeast stearoyl-CoA desaturase mRNA was also reported to decrease when exogenous fatty acids were added to the growth medium (Bossie and Martin, 1989).

Due to the immediacy of the response to the Tween-18:1 treatment, the initial regulation of fatty acid synthesis in tobacco cells probably occurred at the enzymatic level. However, because the inhibition of fatty acid synthesis was observed over several hours it was also possible that the cells would adapt such that the activities of ACCase and fatty acid synthetic enzymes were decreased due to lower enzyme levels. In order to determine if the decreased ACCase was due to the attenuation of enzyme levels, the levels of the biotin carboxylase and biotin carboxyl carrier protein (BCCP) subunits of the plastid ACCase were determined by immuno-blot analysis. Antibodies made to the castor seed biotin carboxylase, and biotin were used to detect the levels of the biotin carboxylase and BCCP subunits of the plastidial ACCase in extracts prepared from control and Tween-18:1 treated tobacco cells. Surprisingly, the levels of both subunits did not change over a 32 hour period in either the control or Tween-18:1 treated cells (Fig. 4-6). The levels of the 200 kD cytosolic form of ACCase were also unaffected by the addition of Tween-18:1 (Fig. 4-6). Furthermore, the levels of

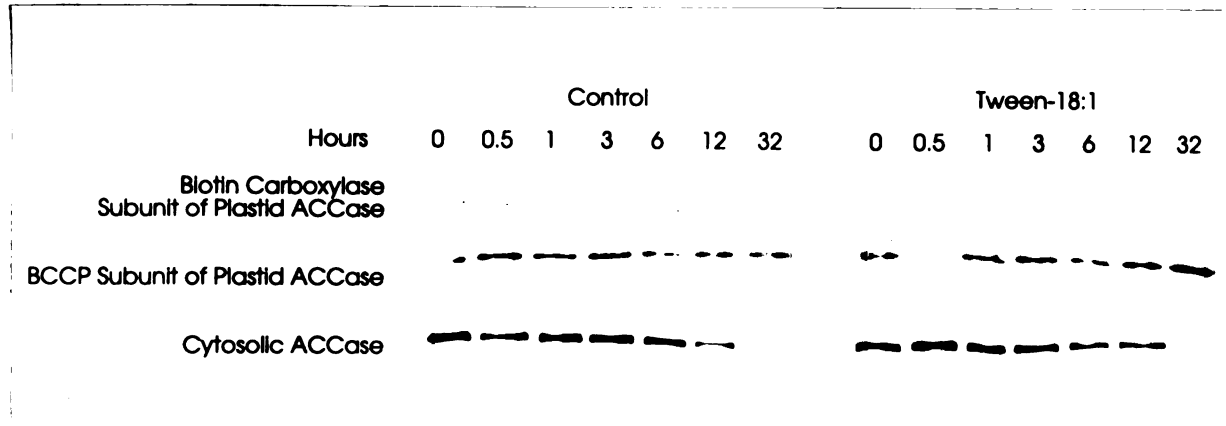


Fig. 4-6. Immuno-blot analysis of ACCase in extracts isolated from control and Tween-18:1 treated cells. 100 mg of total protein extracted from tobacco cells grown in the presence and absence of 900 mM Tween-18:1 were separated on replica SDS-PAGE gels and blotted to nitrocellulose filters. Individual filters were probed with either antibodies made to the cator seed biotin carboxylase subunit of the plastid ACCase to detect biotin carboxylase levels or antibodies made to biotin to detect both the BCCP subunit of the plastid ACCase and the cytosolic ACCase.

two other enzymes involved in fatty acid synthesis, specifically KAS III and stearoyl-ACP desaturase, were also unaffected by the Tween-18:1 treatment (data not shown). These results indicate that the decreased flux through the fatty acid synthetic pathway was not due to a general decrease in FAS enzyme levels, but was more likely due to the inhibition of enzyme activity arising through allosteric or covalent post-translational modifications. However, it can not be ruled out that expression of some specific FAS protein not measured in this study was altered by the Tween-18:1 treatment. Individual filters were probed with either antibodies made to the castor seed biotin carboxylase subunit of the plastid ACCase to detect biotin carboxylase levels or antibodies made to biotin to detect both the BCCP subunit of the plastid ACCase and the cytoplasmic ACCase.

Conclusions

The analysis of the tobacco cell acyl-ACP pools allowed us to develop a model depicting how fatty acid synthesis was being regulated by feedback inhibition. The cumulative data obtained from these experiments indicated that ACCase was being down regulated in response to the addition of exogenous fatty acids. The observed acyl-ACP profiles of the Tween-18:1 treated cells were consistent with what was observed and predicted to occur under conditions where malonyl-CoA would be limiting. Although these studies could not rule out the possibility that multiple components of fatty acid synthase were being coordinately down regulated with ACCase, our data clearly showed that none of the condensing enzymes were being individually inhibited by the feedback regulation. Furthermore, the acyl-ACP analysis indicated that the long chain acyl-ACPs thioesterases were not inhibited during the feedback inhibition of

fatty acid synthesis resulting in the rapid depletion of the long chain acyl-ACP pools. This observation suggest that the long chain acyl-ACPs may not act be the molecules involved in the perception of the feedback inhibition of fatty acid synthesis. The results of the immuno-blot experiments suggested that the feedback inhibition of fatty acid synthesis was not due to an attenuation of ACCase levels, but was probably due to covalent or allosteric modifications of this and possibly other fatty acid synthetic enzymes.

Acknowledgements

I would also like to acknowledge Edgar Cahoon for his help with the lipid analysis and for helping to develop the long and medium chain acyl-ACP analysis. I would like to thank Drs. Dusty Post-Beittenmiller, Keith Roesler, and Mike Walsh for their critical discussion of this manuscript. We also thank Keith Roesler for the biotin carboxylase antibodies, Jan Jaworski and Hee Young Tai for the KAS III antibodies and John Shanklin for the stearoyl-ACP desaturase antibodies. Acknowledgement is also made to the Michigan Agricultural Experiment Station for its support of this work.

Literature Cited

Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:** 911-917

Bossie MA, Martin CE (1989) Nutritional regulation of yeast delta-9 fatty acid desaturase activity. J Bacteriol **171:** 6409-6413.

Clarke SD, Armstrong MK, Jump DB (1990) Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. J Nutr **120:** 225-231.

Cronan JE, Klages AL (1981) Chemical synthesis of acyl thioesters of acyl carrier protein with native structure. Proc Natl Acad Sci USA **78:** 5440-5444.

De Silva NS, Fowler MW (1976) The lipid composition of *Acer pseudoplatanus* cells grown in culture. Phytochemistry **15:** 1735-1740.

Goodridge AG (1985) Fatty acid Synthase in Eukaryotes. *In* DE Vance, JE Vance, eds, Biochemistry of Lipids and Membranes. Benjamin/Cummings Publishing Company Inc.

Henderson TO, McNeil JJ (1966) The control of fatty acid synthesis in Lactobacillus plantarum. Biochem Biophys Res Comm **25:** 662-669.

Jacobs RA, Sly WS, Majerus PW (1973) The regulation of fatty acid biosynthesis in human skin fibroblast. J Biol Chem **248:** 1268-1276.

Jaworski JG, Post-Beittenmiller D, Ohlrogge JB (1993) Acetyl-acyl carrier protein is not a major intermediate in fatty acid biosynthesis in spinach. Eur J Biochem **213:** 981-987.

Jiang P, Cronan JE (1994) Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. J Bacteriol **176:** 2814-2821.

Kamiryo T, Numa S (1973) Reduction of the acetyl coenzyme A carboxylase content of Saccharomyces cerevisiae by exogenous fatty acids. FEBS Lett **38:** 29-32.

Kim KH (1983) Regulation of acetyl-CoA carboxylase. Curr Top Cell Regul **22:** 143-172.

Kitajima K, Tashiro S, Numa S (1975) Acetyl-CoA carboxylase in cultured hepatocytes: Effects of exogenous fatty acids on the content, synthesis and degradation of the enzyme. Eur J Biochem **54:** 373-383.

Kuo TM, Ohlrogge JB (1984) Acylation of plant acyl carrier protein by acyl acyl carrier protein synthetase from *E. coli*. Arch Biochem Biophys **230:** 110-116.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurements with the folin phenol reagent. J Biol Chem **193:** 265-275.

Morrison WR, Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride methanol. J Lipid Res **5:** 600-608.

Nikawa J, Tanabe T, Ogiwara H, Shiba T, Numa S (1979) Inhibitory effects of long chain acyl-CoA analogues on rat liver acetyl-CoA carboxylase. FEBS Lett **102:** 223-278.

Nunn WD, Kelly DL, Stumfall MY (1977) Regulation of fatty acid synthesis during the cessation of phospholipid biosynthesis in *Escherichia coli.* J. Bacteriology **132:** 526-531.

Ohlrogge JB, Jaworski JG, Post-Beittenmiller D (1993)*De novo* fatty acid biosynthesis. *In* TS Moore ed, Lipid Metabolism in Plants. CRC Press Inc., Ann Arbor, MI, USA pp 3-32.

Ohlrogge JB, Shine WE, Stumpf PK (1978) Fat Metabolism in higher plants. Characterization of plant acyl-ACP and acyl-CoA hydrolases. Arch Biochem Biophys **189:** 306-391.

Post-Beittenmiller MA, Jaworski JG, Ohlrogge JB (1992a) Regulation of lipid synthesis in castor seeds: Analysis of the *in vivo* acyl-acyl carrier protein pool. *In* SL MacKenzie and DC Taylor eds. Seed oils for the future. AOCS Press, Champaign, IL, USA, pp 44-51.

Post-Beittenmiller D, Roughan G, Ohlrogge JB (1992b) Regulation of plant fatty acid biosynthesis: Analysis of acyl-CoA and acyl-acyl carrier protein substrate pools in spinach and pea chloroplast. Plant Physiol. **100:** 923-930.

Post-Beittenmiller MA, Jaworski JG, Ohlrogge JB (1991) *In vivo* pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem **266:** 1858-1865.

Roughan G, Nishida I (1990) Concentrations of long chain acyl-acyl carrier proteins during fatty acid synthesis by chloroplasts isolate from pea (*Pisum sativum*), safflower (*Carthamus tinctoris*), and amaranthus (*Amaranthus lividus*) leaves. Arch Biochem Biophys **276:** 38-46.

Sabitis JE, Powell GL (1976) Acyl carrier protein metabolism and regulation of fatty acid biosynthesis by *Lactobacillus plantarum.* J Biol Chem **251:** 4706-4712.

Shintani DK, Ohlrogge JB (1994) The characterization of a mitochondrial acyl carrier protein isoform isolated from *Arabidopsis thaliana.* Plant Physiology **104:** 1221-1229.

Soll J, Roughan G (1982) Acyl-acyl carrier protein pool sizes during-steady-state fatty acid synthesis by isolated spinach chloroplast. FEBS Letters **146:** 189-192.

Stapleton SR, Jaworski JG (1984) Characterization and purification of malonyl-coenzyme A: [acyl carrier protein] transacylases from spinach and *Anabaena variabilis*. Biochem Biophys Acta **794:** 204-248.

Terzaghi WB (1986a) A system for manipulating the membrane fatty acid composition of soybean cell cultures by adding Tween-fatty acid esters to their growth medium. Plant Physiol **82:** 771-779.

Terzaghi WB (1986b) Metabolism of Tween-fatty acid esters by cultured soybean cells. Plant Physiol **82**: 780-786.

Terzaghi WB, Roth J, Keim P, Honeycutt RJ, Lark KG (1985) A somatic genetic system for soybean. *In* M Freeling, ed, Plant Genetics. Alan R Liss, Inc, New York, pp 771-789.

Chapter 5

Antisense and over-expression of biotin carboxylase in tobacco leaves

Abstract

Several *in vivo* studies have implicated the plastid acetyl-CoA carboxylase as an important regulatory enzyme in fatty acid synthesis. The plastid ACCase is present as a heteromeric complex that is composed of four different protein subunits, specifically biotin carboxylase, biotin carboxyl carrier protein, and the α- and β-subunits of the carboxyltransferase. In order to test our assumptions of ACCase's role in regulating fatty acid synthesis and also to gain some insight into the subunit organization of this heteromeric enzyme complex, biotin carboxylase expression was altered in transgenic plants. Tobacco plants were transformed with antisense- and over-expression tobacco biotin carboxylase constructs which resulted in the generation of plants with biotin carboxylase levels ranging from 10% to 1000% of wild type levels. A comparison of leaf biotin carboxylase and BCCP levels in plants showing elevated and decreased biotin carboxylase expression, revealed that these two subunits of the plastid ACCase are not maintained in a strict stoichiometric ratio. Tobacco plants containing elevated and moderate decreases in leaf biotin carboxylase were indistinguishable from wild type plants. However, one plant, with only 10% of wild type biotin carboxylase levels, showed severely retarded growth when grown under low light conditions and significantly lower leaf fatty acid content than wild type plants. Furthermore, the homozygous progeny of this plant exhibited aberrant floral development.

Introduction

Fatty acid synthesis is central to the growth and development of most organisms. Along with the central role that this pathway plays in the synthesis of membrane lipid precursors, fatty acid synthesis is also necessary for the synthesis of seed storage oils and waxes which are valuable agronomic commodities.

Although much is known about the biochemistry involved in the synthesis of fatty acids, little is understood about how this pathway is regulated. However recently, several *in vivo* studies have implicated the plastid localized acetyl-CoA carboxylase (ACCase) as an important regulatory enzyme in fatty acid synthesis. First, careful measurements of acyl-acyl carrier protein (acyl-ACP) pools in light and dark treated spinach leaves and chloroplasts indicated that the plastid ACCase is involved in the light activation of fatty acid synthesis (Post-Beittenmiller et al., 1991, 1992). It was also demonstrated through acyl-ACP analysis that the plastid ACCase is involved in the product feedback inhibition of fatty acid synthesis in tobacco suspension cultures (Shintani and Ohlrogge, 1995). Furthermore, using specific inhibitors of the maize plastid ACCase, Page et al. (1994) demonstrated that ACCase exerts a high degree of flux control over fatty acid synthesis in maize leaves. These findings agree with observations from animal, fungal, and bacterial systems that implicate ACCase as an important enzyme involved in the regulation of fatty acid synthesis in those organisms (Goodridge, 1985; Kamiryo and Numa, 1973; Magnuson et al., 1993).

Due to its important role in regulating plant fatty acid synthesis, the plastid ACCase has become the subject of intense interest. This interest has led to the discovery that in dicot and non-gramineae monocot plants, the plastid ACCase exists as a heteromeric enzyme complex composed of four independent

polypeptides corresponding to the biotin carboxyl carrier protein, the biotin carboxylase, and the α- and β-subunits of carboxyltransferase (Sasaki et al.,1994; Alban et al., 1994; Shorrosh et al., 1995 and manuscript submitted; Choi et al., 1995).

Recently, the genes coding for the biotin carboxylase, BCCP, and the α- and β-subunits of carboxyltransferase have been cloned from plants (Sasaki et al.,1994; Shorrosh et al., 1995 and in press; Choi et al., 1995). This achievement has now made it possible to test assumptions about ACCase's regulatory role in plant fatty acid synthesis. It is now possible to modify the level of the plastid ACCase by altering expression of genes coding for one or more of the ACCase subunits in transgenic plants. If ACCase has a strong regulatory influence on the flux through fatty acid synthase, then changes in ACCase activity should significantly alter rates of fatty acid synthesis.

We have previously reported the cloning of a tobacco cDNA clone corresponding to the biotin carboxylase subunit of the plastid ACCase (Shorrosh et al., 1995). In the present study, the biotin carboxylase cDNA was expressed in the sense and antisense orientation behind a strong constitutive promoter in transgenic tobacco plants. One goal of this work was to determine what effect altering biotin carboxylase level would have on ACCase's ability to regulate the flux through fatty acid synthesis. Additionally, we were interested in determining how the expression of other subunits of the plastid ACCase would be effected by altering levels of biotin carboxylase.

Materials and Methods

Construction of biotin carboxylase plant expression constructs and transformation of these constructs into tobacco

The tobacco biotin carboxylase cDNA clone (Shorrosh et al., 1995) was subcloned in the sense and antisense orientation as a *Pst*I fragment in place of the *adh*I intron I *Pst*I fragment in the plant gene expression vector p1079. The p1079 vector contained the promoter for the 35s transcript of the cauliflower mosaic virus (CaMV) that was engineered to contain two copies of the CaMV 35s enhancer element and the nopoline synthase 3' terminator sequences. The double CaMV 35S promoter of p1079 was constructed by subcloning the *Hinc*II *EcoR*V fragment corresponding to the CaMV 35S enhancer region of the CaMV 35S promoter into the *Hinc*II site of the CaMV 35S promoter. The biotin carboxylase sense and antisense expression constructs were subcloned as *Xba*I fragments into the *Xba*I site of pBIN19 (Bevin, 1984). The resulting over- and antisense-expression/*Agrobacterium* binary vector constructs were designated as p0719S and p0719A respectively. These constructs were introduced into the *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. (1983) by direct transformation as describe by An et al. (1988). Tobacco (*Nicotiana tabacum*) leaf disc were transformed as described by Rogers et al. (1986).

Affinity purification of caster bean biotin carboxylase antibodies to *Escherichia coli (E. coli)* expressed tobacco biotin carboxylase.

The DNA sequence from the tobacco biotin carboxylase cDNA clone corresponding to the mature biotin carboxylase protein was amplified by polymerase chain reaction (PCR) and subcloned into pET15b (Novagen, Madison, WI) as described by Shorrosh et al. (1995). The biotin carboxylase

was expressed in *E. coli* and purified as described by Shorrosh et al. (1995).

Approximately 4 mg of the purified *E. coli*-expressed biotin carboxylase protein in 100 mM MES pH 5.8 was reacted with 1 ml of Affigel-10 (BioRad, Richmond, CA) for 30 minutes at 4°C on a rocking platform, after which the gel matrix was collected by centrifugation at 10,000 g for 10 minutes. The supernatant was then reacted a second time with 1 ml of Affigel-15 (BioRad, Richmond, CA) overnight at 4°C on a rocking platform. The reacted Affigel-10 and Affigel-15 were then combined and washed sequentially with 10 column volumes of 10 mM Tris pH7.5, 10 column volumes of 100 mM citrate pH 2.5, 10 column volumes of 10 mM Tris pH 8.8, and 10 column volumes of TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.02% sodium azide).

Ammonium sulfate was added to 50% saturation to 5 ml of the rabbit antiserum against caster bean biotin carboxylase (Roesler et al., 1995). The ammonium sulfate pellet was then resuspended in 2.5 ml of TBS (10 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide). The solution was desalted on a PD-10 desalting column (Pharmacia) equilibrated with TBS and bound to the biotin carboxylase affinity column overnight at 4°C on a rocking platform. The affinity column was washed with 10 column volumes of TBST and the antibody was eluted with 10 column volumes of 100 mM citrate pH 2.5. The pH of eluted fractions was neutralized by adding 1 M Tris pH 8.8.

Immuno-blot analysis of tobacco leaf extracts.

Tobacco leaves were immediately frozen in liquid nitrogen after being harvested. Approximately 1 gm of leaf material was then ground to a fine powder in liquid nitrogen. The frozen ground tissue was then homogenized in 3 ml of 50 mM HEPES-KOH, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF),

1 mM benzamidine using a Brinkmann polytron at the highest setting. SDS was then added to the homogenate to a final concentration of 1%. This mixture was then incubated in a boiling water bath for 5 minutes. The homogenate was clarified by centrifuging at 10,000 g for 20 minutes at 15°C. The protein concentration of each leaf extract was determine as described by Bradford (1976). One hundred µg protein aliquots of each leaf extract were fractionated on 10% SDS-PAGE gels as described by Laemmli (1970). Proteins were then transferred to nitrocellulose filters as described by Post-Beittenmiller et al. (1989) and the filters were probed with either a 1/250 dilution of the affinity purified biotin carboxylase polyclonal antibodies or a 1/2000 dilution of biotin antibodies as described by Roesler et al. (1995).

Fatty acid Analysis

Approximately 50 mg of fresh leaf tissue was homogenized in 1 ml of 50 mM HEPES, 5 mM DTT, 1 mM PMSF, 1 mM benzamidine plus 25 µg heptadecanoic acid (17:0). Total lipid was extracted from the homogenate as described by Bligh and Dyer (1959). The extracted lipids were concentrated under nitrogen and fatty acid methyl esters (FAMEs) were prepared by resuspending the dried lipid in 1 ml of 10% boron trichloride/methanol (Sigma). This mixture was heated at 90*C for 30 minutes. The methylation reaction was stopped by adding of 1 ml of water and FAMEs were extracted 2 times with 2 ml of hexane. Leaf FAMEs were analyzed by gas chromatography (GC) with a Hewlett-Packard 5890 GC using a 30 m x 0.25 mm i.d. DB 23 column (J&W Scientific, Rancho Cordova, CA) with a oven temperature programmed from 180*C (10 minute hold) to 230*C at 3*C/minute with a column head pressure of 200 kPa of helium.

Results and Discussion

Over- and antisense-expression of biotin carboxylase resulted in the generation of transgenic tobacco showing a wide range of leaf biotin carboxylase expression levels.

The full length tobacco biotin carboxylase cDNA (Shorrosh et al., 1995) was subcloned in the sense and antisense orientation behind the CaMV 35S promoter. The tobacco biotin carboxylase over- and antisense-expression constructs, designated p0719S and p0719A respectively, were used in the *Agrobacterium* mediated transformation of tobacco leaf disc. These transformations yielded 24 independent kanamycin resistant plants from each construct.

The levels of biotin carboxylase protein expression in the leaves of p0719S and pO719A transformants were determined by immuno-blot analysis using antibodies affinity purified to the tobacco biotin carboxylase. A broad range of biotin carboxylase expression levels was observed in the leaves of both the sense (p0719S) and antisense (p0719A) transformants (Fig. 1A and B). Leaf biotin carboxylase protein was significantly elevated over wild type levels in several of the plants transformed with the p0719S biotin carboxylase over-expression construct. These transformants contained biotin carboxylase levels that ranged from 2 to 5 fold over wild type levels (i.e. S2 and S6, Fig. 1A). Northern blot analysis of RNA isolated from the leaves of these p0719S plants indicated that biotin carboxylase mRNA levels also increased in correlation with the observed increase in biotin carboxylase protein (data not shown). None of the 24 independent p0719S transformants were found with more than a five fold increase in leaf biotin carboxylase levels.

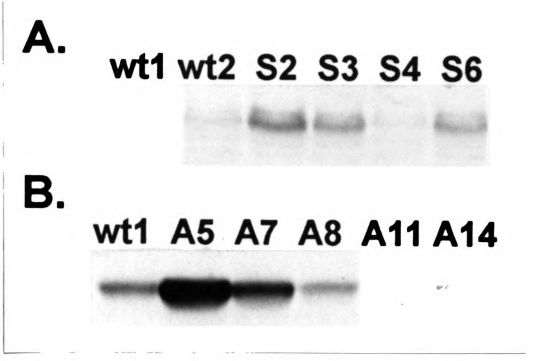


Figure 5-1. Biotin carboxylase levels in transgenic tobacco leaves. One hundred μg aliquots of leaf protein extract, isolated from plants transformed with (A) the p0719S over-expression construct and (B) the p0719A antisense construct, were fractionated on 10% SDS-PAGE gels and blotted to nitrocellulose. Biotin carboxylase was detected using affinity purified antibodies to the tobacco biotin carboxylase.

Decreased biotin carboxylase protein levels were observed in approximately 30% of the plants transformed with the p0719A construct. Of these plants, most showed decreases in leaf biotin carboxylase levels that ranged from 30% to 50% of wild type levels (i.e. A14 and A15, Fig. 1B). However, of the twenty-four independent p0719A transformants, only one plant was severely deficient in leaf biotin carboxylase. This transformant, designated as A11, showed a 90% decrease in leaf biotin carboxylase protein relative to wild type plants (Fig. 1B). Northern blot analysis of RNA isolated from the leaves of the under expressing p0719A plants using sense and antisense strand specific RNA probes showed that levels of biotin carboxylase mRNA were almost undetectable while a large accumulation of antisense RNA was observed (data not shown). The observation that no plants were found containing biotin carboxylase levels lower than 10% of wild type levels may suggest that biotin carboxylase must be present at a certain minimal level to maintain the viability of the plant. Furthermore, the low frequency of p0719A transformants containing biotin carboxylase levels lower than 50% of wild type levels could also be explained by the hypothesis that decreasing biotin carboxylase levels to very low levels is detrimental to the plant.

While the expected increases and decreases in leaf biotin carboxylase levels were observed in the majority of the plants transformed with the over- and antisense-expression constructs, a small proportion of the p0719S and p0719A transformants gave unexpected patterns of biotin carboxylase expression. For example, of the 24 p0719S transformants, one plant (i.e S4, Fig 1A), contained decreased levels of leaf biotin carboxylase protein. The decrease in biotin carboxylase expression in these plants transformed with an over-expression construct was most likely due to transgene inactivation. Similar results were first

observe during attempts to over-express the chalcone synthase gene using the CaMV 35S promoter in petunia (Napoli et al., 1990; van der Krol et al., 1990). Since then this phenomena has been observed by several groups and transgene inactivation is now being used as an alternative to antisense approaches as a means to decreasing gene expression in plants (Finnegan and McElroy, 1994 and references within).

Surprisingly, several plants transformed with the antisense biotin carboxylase construct contained elevated levels of leaf biotin carboxylase. Two "antisense" transformants, A5 and A7 (Fig 1B), were found to contain biotin carboxylase levels that were 5 to 10 fold higher than those measured in wild type plants. These increased levels of biotin carboxylase expression rivaled and surpassed those observed in the highest expressing p0719S plants (i.e. S2 and S6 see Fig. 1A). Biotin carboxylase over-expression was also observed at a low frequency in tobacco suspension cells independently transformed with the p0719A antisense construct (data not shown). At present, we do not have an explanation for over-expression of biotin carboxylase occurring from plants transformed with an antisense construct. However, because rearrangements of the T-DNA have been reported to occur upon the integration during *Agrobacterium* mediated transformation, one possible explanation could involve the rearrangement of the antisense construct. Alternatively, the integration of the p0719A T-DNA could have occurred such that the transgene was oriented in the sense orientation behind a strong endogenous promoter. Extensive genomic DNA analysis would be needed to distinguish between these and other possibilities.

Increases and decreases in biotin carboxylase levels had no effect on the expression of the BCCP subunit of ACCase

To determine if the levels of the different subunits of the plastid ACCase were maintained in a strict stoichiometric ratio, the levels of BCCP were determined in the leaves of plants with altered levels of biotin carboxylase. Several reports indicate that the expression of individual subunits of various chloroplast complexes are coordinately regulated with the levels of other subunits or components of the same complex. For example, the expression of the ribulose 1,5-bisphosphate oxygenase/carboxylase small subunit (SSU-RUBISCO) was found to be dependent on the levels of the large subunit of RUBISCO (LSU-RUBISCO) such that uncomplexed subunits were rapidly degraded (Schmidt and Miskind, 1983). Furthermore, antisense-expression of the SSU-RUBISCO in tobacco resulted in the coordinate decrease of the LSU-RUBISCO (Rodermel et al., 1988). It has also been reported that in chlorophyll biosynthetic mutants, light-harvesting chlorophyll a/b proteins (LHCPs) are rapidly degraded due to their inability to assemble the correct pigment/protein complex (Plumley and Schmidt, 1995). The stoichiometric ratios of RUBISCO and LHC protein subunits are maintained by a post-translational mechanism where excess subunits are proteolyticly degraded.

To determine if the subunits of the plastid ACCase are coordinately regulated in a similar fashion to the RUBISCO or LHC subunits, BCCP levels were measured in the leaves from wild type plants and the two transgenic plants containing the highest and lowest levels of leaf biotin carboxylase, specifically A5 and A11. The studies revealed that the BCCP levels were approximately equivalent in A5 and A11 leaf extracts, but were slightly higher in wild type leaves (Fig. 2). Although it is not clear why wild type plants expressed BCCP

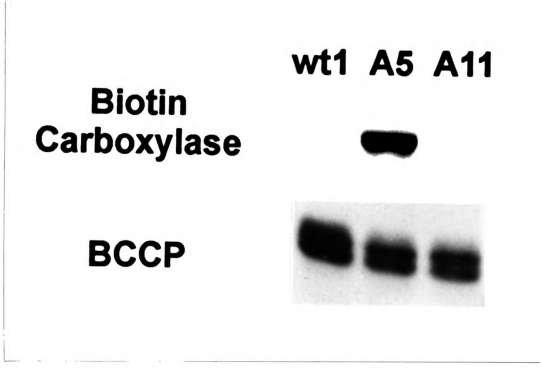


Figure 5-2. Comparison of leaf BCCP and biotin carboxylase levels in transgenic tobacco leaves containing altered levels of biotin carboxylase. One hundred μg aliquots of leaf protein extract, isolated from wild type, A5, and A11 plants were run in duplicate on 10% SDS-PAGE gels and blotted to nitrocellulose. Biotin carboxylase and BCCP were detected on separate blots using affinity purified antibodies to the tobacco biotin carboxylase and antibodies to biotin.

levels at slightly higher levels than either of the transgenic lines, these results indicate that BCCP levels were not effected by either large increases or large decreases in biotin carboxylase levels. Therefore, we conclude that biotin carboxylase levels are not coordinately maintained in a strict stoichiometric ratio with the BCCP subunit of the plastid ACCase. The presence of excess biotin carboxylase subunits in over-expressing plants without an increase in BCCP levels indicates that the plastid must be able to tolerate uncomplexed ACCase subunits. It appears that the mechanism maintaining the strict coordination of RUBISCO and LHC subunits does not apply to ACCase.

Plants containing extremely low levels of biotin carboxylase exhibit a stunted phenotype when grown under low light conditions

While plants exhibiting high and moderately low levels of leaf biotin carboxylase were phenotypically indistinguishable from wild type plants, the growth and development of the A11 transformant, which contained very low levels of leaf biotin carboxylase, was severely retarded when grown under low light conditions (i.e. approximately 45 $\mu E/m^2$) (Fig. 3). In addition to the stunted growth, the leaves of the A11 plant were slightly chlorotic and less succulent than the leaves of wild type plants that were grown under the same low light conditions. However, when the A11 plant was transferred to higher light conditions (i.e. >250 $\mu E/m^2$), it grew vigorously and caught up developmentally with wild type plants.

The stunted growth under low light conditions of the A11 plant was also observed in the kanamycin resistant progeny of the self crossed primary transformant suggesting that the antisense trait was segregating as a single genetic locus (data not shown). The degree of stunting appeared to be more

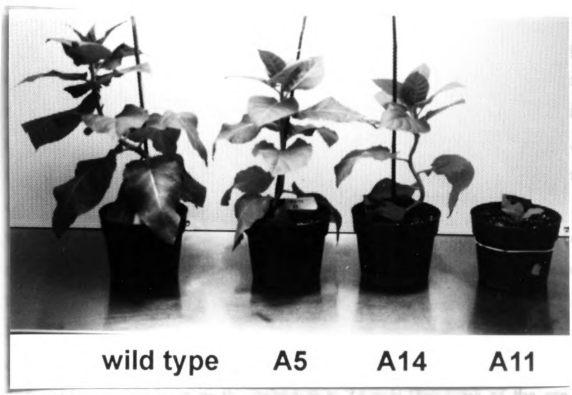


Figure 5-3. Growth of wild type plants and plants expressing increased and decreased levels of biotin carboxylase under low light conditions. A wild type plant and primary p0719A transformants were grown in a growth chamber at 25 °C under constant illumination of 45 $\mu E/m^2$.

severe in 1/4 of the second generation plants indicating that the antisense phenotype was additive and possibly due to a gene dosage effect. When the second generation A11 plants were grown under higher light conditions, the stunted phenotype was again not observed (data not shown).

Decreased fatty acid content was associated with A11 plants grown under both high and low light conditions.

Although a dwarf phenotype was associated with plants exhibiting large decreases in leaf biotin carboxylase levels, it was not clear if this phenotype was associated with decreased fatty acid synthesis due to lower ACCase activity in these plants. Unfortunately, due to the instability of the ACCase and FAS complexes, it was difficult to accurately access rates of fatty acid synthesis and chloroplast ACCase activity in isolated tobacco chloroplast or leaf extracts. It was however possible to measure leaf fatty acid content by gas chromatography analysis. Such analysis allows for an indirect determination of the over-all effect that changes in biotin carboxylase levels had on membrane lipid metabolism. Fatty acid analysis was performed on leaves from plants expressing increased, decreased,
and wild type levels of biotin carboxylase. Leaves that were of the same approximate size were sampled from plants grown under low and high light conditions.

These studies revealed that A11 leaves synthesized less fatty acid on a fresh weight basis than the leaves of plants expressing wild type or elevated levels of biotin carboxylase. This decrease in A11 leaf fatty acid content was observed in plants grown under high and low light conditions. The stunted A11 plants grown under low light conditions had a 26% decrease in leaf fatty acid

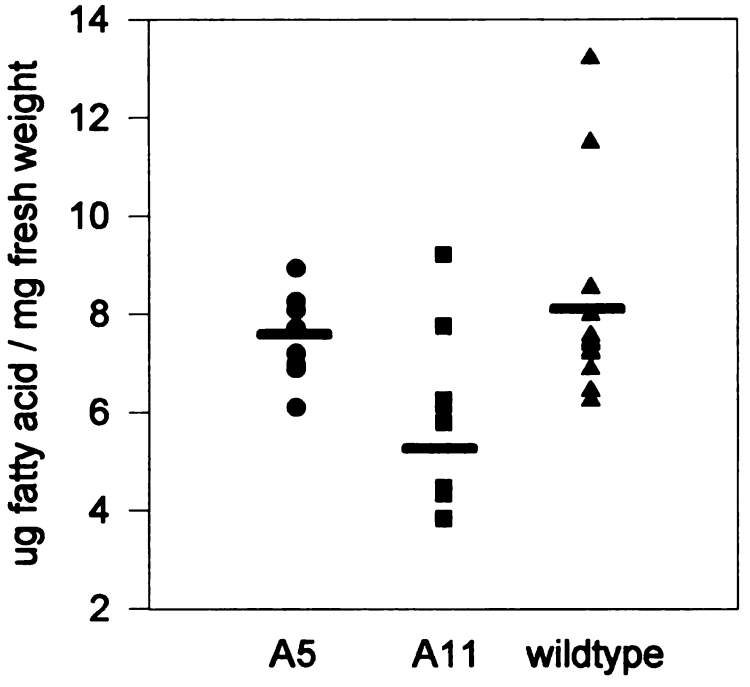
content when compared to wild type plants grown under the same conditions (Fig. 4A). Although the A11 plants were phenotypically indistinguishable from wild type plants when grown under higher light conditions, a modest, but statistically significant, 8% decrease in fatty acid content was measured in the leaves of these plants (Fig. 4B). Although the fatty acid content varied in different leaves sampled from the same plant, according to the Wilcoxon-Mann-Whitney statistical test, the leaf fatty acid content of A11 plants, grown under both light conditions, was significantly different (i.e. confidence level > 99%) from that of wild type plants grown under the same conditions. This same statistical test also indicated that the leaf fatty acid content of A5, the transgenic plant showing the highest levels of biotin carboxylase, was not significantly different from wild type plants when grown under either conditions. Additionally, the leaf fatty acid content of plants showing only modest decreases in leaf biotin carboxylase levels were also not significantly different from wild type plants (data not shown).

The biotin carboxylase subunit of ACCase may be present in excess in wild type tobacco leaves

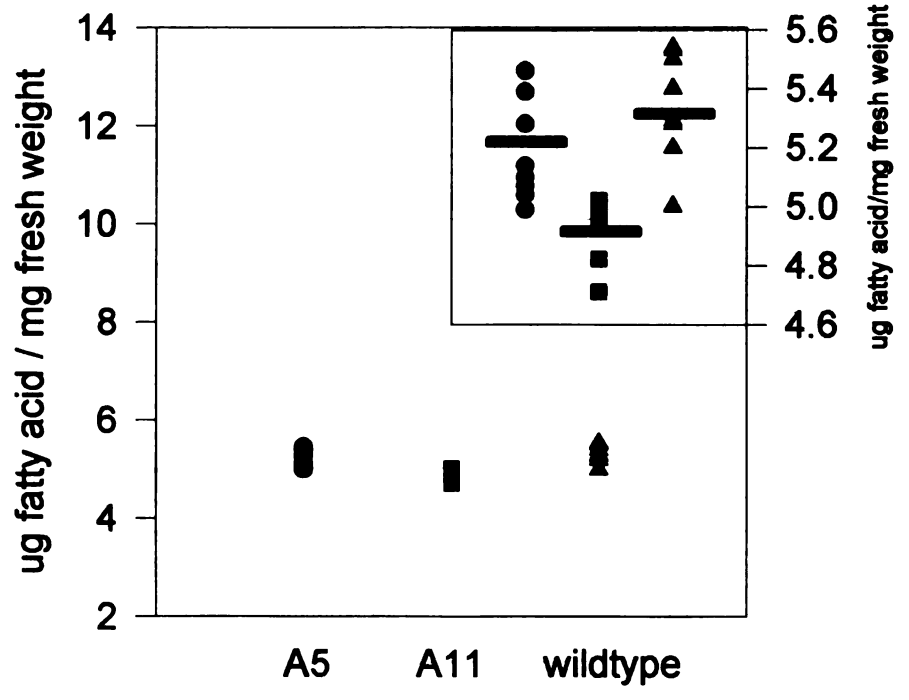
The observation that biotin carboxylase levels could be decreased to as low as 50% of wild type level without effecting the levels of BCCP, fatty acid content or growth rate suggest that biotin carboxylase may be present in excess in wild type plants. It was only after biotin carboxylase levels were decreased by 90% in A11 plants that changes in fatty acid content and growth rate were observed. This result is consistent with those of antisense experiments using the genes encoding pyrophosphate:fructose-6-phosphate phosphotransferase, fructose-1,6-bisphosphatase, and citrate synthase. In these studies, a greater than

Figure 5-4. Leaf fatty acid content of wild type, A5 and A11 plants grown under (A) low light and (B) high light conditions. Approximately 50 μg leaf samples were taken from leaves of plants grown under 45 $\mu E/m^2$ and 300 $\mu E/m^2$ intensity light. Total lipid was extracted from leaves with 20 or 25 μg of heptadecanoic acid (17:0) added as an internal standard. The total lipid was then transesterified to fatty acid methylesters (FAMEs) using BCl_3 in methanol. FAMEs were then analyzed by gas chromatography. (A) Leaf fatty acid content of plants grown under constant illumination by 45 $\mu E/m^2$ light. (B) Leaf fatty acid content of plants grown under constan illumination by 300 $\mu E/m^2$ light. The insert if (B) shows the same data expanded on a different scale. Horizontal bars represent the mean value of the combined samples from each plant. A5, A11, and wild type plants are represnted by closed circles, closed squares and close triangles respectively.

A.


14
12
10
8
6
4
2
ug fatty acid / mg fresh weight
A5
A11
wildtype

B.


14
12
10
8
6
4
2
ug fatty acid / mg fresh weight
5.6
5.4
5.2
5.0
4.8
4.6
ug fatty acid/mg fresh weight
A5
A11
wildtype

80% decrease in enzyme levels was needed before changes in metabolism or phenotype were detected (KoBmann et al., 1994; Hajirezaei et al., 1994; Landschutze et al, 1995). According to metabolic control theory, in instances where large changes in enzyme levels are needed to alter flux through biosynthetic pathway, these enzymes are thought to exert little control over pathway flux (Kacser and Porteous, 1987). However, the results from antisense studies using genes coding for enzymes with previously determined regulatory importance again showed that large decreases in enzyme levels were necessary for altered metabolism. This apparent inconsistency has recently been addressed by Stitt and Sonnewald (1995). They suggest that, under many circumstances, the principles of metabolic control theory do not apply. Plants appear to have multiple mechanisms to compensate for deficiencies with a metabolic pathway such that control is usually shared between several enzymes. Furthermore, several regulatory enzymes operate at well below their maximum activity which allows these enzymes to rapidly respond to changes in metabolism.

Homozygous p0719A-11 plants had abnormal floral development

A11 plants grown under green house conditions were allowed to flower and go to seed. Approximately 1/4 of the kanamycin resistant progeny from the A11 self cross contained mature flowers that were male sterile due to aberrant anther development (Fig. 5). In place of anthers, these plants contained "petal-like" organs attached to the ends of their stamens. The filament portion of the stamen appeared normal as did all of the other floral organs of these plants. These plants were still fertile and did produce seed when crossed with pollen from other plants.

Although it is not clear whether this abnormal flower morphology was due

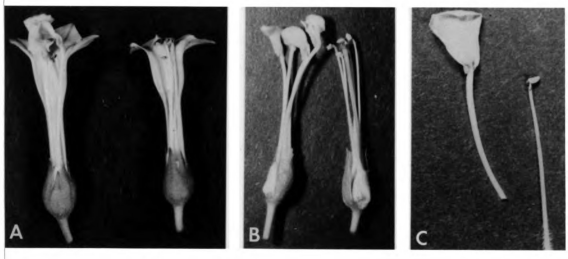


Figure 5-5 Plants that are homozygous for the A11 transgene locus show abnormal floral development. In photographs (A) through (C), tissues harvested from A11 plants are on the left and tissues harvested from wild type plants are on the right. Panel (A) shows whole flowers with the front petals removed. Panel (B) contains stamens dissected from A11 and wild type flowers. Panel (C) contains flowers with all petals removed.

the decrease in biotin carboxylase levels or if it was due to the insertion of the p0719A T-DNA into a gene involved in floral development, alterations in different metabolic pathways have been implicated in altering floral development. For example, mutations in lipid metabolism have resulted in *Arabidopsis* male sterility. McConn and Browse (1993), reported that plants lacking trienoic fatty acids were male sterile due to their inability to produce jasmonic acid which is needed for pollen maturation. Furthermore, severe decreases in citrate synthase in potato plants transformed witha citrate synthase antisense construct was associated with inhibition of flower formation (Landschutze et al., 1995). The ovaries of these plants failed to develop past a early stages of flower development. It may be possible that the aberrant stamen development in A11 plants is due to insufficient fatty acid synthesis associated with the decrease in biotin carboxylase levels.

Conclusion

Several new insights into the regulation and complex structure of the plastid ACCase have been revealed through these studies. First, it appears that the levels of biotin carboxylase are not maintained in a strict stoichiometric ratio with BCCP and presumably other subunits of the plastid ACCase. As observed in plants expressing high levels of biotin carboxylase, the tobacco chloroplast were able to tolerate the accumulation of uncomplexed subunits of ACCase. These results indicate that the formation and maintenance of the plastid ACCase complex differs from other plastid localized protein complexes such as RUBISCO and LHC.

Second, transgenic tobacco plants were able to tolerate 50% decreases in biotin carboxylase levels without effecting growth or leaf fatty acid content.

These results suggest that the biotin carboxylase subunit of ACCase or the ACCase complex may be present in excess in wild type leaves. However, under low light conditions, severe retardation of growth was observed in the A11 plant which expressed very low levels of leaf biotin carboxylase. Analysis of the leaf fatty acid content of the A11 transformant suggested that the stunted phenotype was associated with these plants inability to maintain sufficient rates of fatty acid synthesis. In addition, aberrant stamen development was observed in the flowers of homozygous A11 plants. Because only one transformant was generated that exhibited these unusual phenotypic traits, it is not certain if these traits can be directly associated with decreased biotin carboxylase levels. It is possible that the atypical patterns of growth and development associated with the A11 transformant are due to the insertion of the p0719A T-DNA into region of the tobacco nuclear genome which resulted in pleiotropic events leading to these phenotypic changes.

Acknowledgements

We would like to thank Wan-Ling Chu and Pam Green for the generous gift of the p1079 plasmid.

Literature Cited

Alban C, Baldet P, Douce R (1994) Localization and characterization of two structurally different forms of acetyl-CoA carboxylase in young pea leaves, of which one is sensitive to arloxyphenoxypropinate herbicides. Biochem J **300:** 557-565.

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. *In* Plant Molecular Biology Manual, SB Gelvin and RA Schilperoot eds, Kulwer Academic Publishers, pp A3: 1-19.

Bevin M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res **12:** 8711-8727.

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:** 911-917.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248-254.

Choi JK, Yu F, Wurtele ES, Nikolau BJ (1995) Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of the chloroplastic acetyl-CoA carboxylase. Plant Physiol **109:** 619-624.

Finnegan J, McElroy D (1994) Transgene inactivation: Plants fight back. Bio/technology **12:** 883-888.

Goodridge AG (1985) Fatty acid synthase in eukaryotes. *In* Biochemistry of lipids and Membranes, DE Vance, JE Vance, eds, Benjamin/Cummings Publishing Company, Inc, Menlo Park, CA, pp 143-180.

Hajirezaei M, Sonnewald U, Viola R, Carlisle S, Dennis D, Stitt M (1994) Transgenic potato plants with strongly decreased expression of pryophosphate:fructose-6-phosphate posphotransferase show no visible phenotype and only minor changes in metabolic fluxes in their tubers. Planta **192:** 16-30.

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir-* and T-region of the *Agrobacterium tumefaciens* Ti plasmid. Nature **303:** 179-180.

Kacser H, Porteous JW (1987) Control of metabolism: What do we have to measure. Trends Biochem Sci **12:** 14

Kamiryo T, Numa S (1973) Reduction of acetyl coenzyme A carboxylase content of *Saccharomyces cerevisae* by exogenous fatty acids. FEBS Lett. **38:** 29-32.

KoBmann J, Sonnewald U, Willmitzer L (1994) Reduction of the chloroplastic fructose-1,6-bisphosphatase in transgenic potato plants impairs photosynthesis and potato growth. The Plant Journal **6:** 637-650.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

Landschutze V, Willmitzer L, Muller-Rober B (1995) Inhibition of flower formation by antisense repression of mitochondrial citrate synthase in transgenic potato plants leads to a specific disintegration of ovary tissues of flowers. EMBO J **14:** 660-666.

Magnuson K, Jackowski SJ, Rock CO, Cronan JE (1993) Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiological Reviews: **57:** 522-542.

McConn MM, Browse JA (1993) A mutant of Arabidopsis thaliana deficient in trienoic fatty acids is male sterile. Proceeding of the 1993 Plant Lipid Symposium, National Plant Lipid Cooperative.

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologoues genes *in trans*. The Plant Cell **2:** 279-289.

Page RA, Okada S, Harwood JL (1994) Acetyl-CoA carboxylase exerts strong flux control over lipid synthesis in plants. Biochimica et Biophysica Acta **1210:** 369-372.

Plumley FG, Schmidt GW (1995) Light-harvesting chlorophyll A/B complexes - interdependent pigment synthesis and protein assembly. The Plant Cell **7:** 689-704.

Post Beittenmiller MA, Schmid KM, Ohlrogge JB (1989) Expression of holo and apo forms of the spinach acyl carrier protein-I in leaves of transgenic tobacco plants. The Plant Cell **1:** 889-899.

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) In vivo pools of free and acylated acy carrier proteins in spinach: Evidense for sites of regulation of fatty acid biosynthesis. J Biol Chem **266:** 1858-1865.

Post-Beittenmiller D, Roughan G, Ohlrogge JB (1992) Regulation of plant fatty acid biosynthesis: Analysis of acyl-Coenzyme A and acyl-acyl carrier protein substrate pools in spinach and pea chloroplast. Plant Physiol **100:** 923-930.

Rodermel SR, Abbott MS, Bogorad L (1988) Nuclear-organelle interactions: Nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell **55:** 673-681.

Roesler KR, Savage LJ, Shintani DK, Shorrosh BS, Ohlrogge JB (1995)-Co-purification, co-immunopreciptation, and coordinate expression of acetyl-CoA carboxylase activity, biotin carboxylase, and biotin carboxyl carrier protein of higher plants. Planta **198:** (in press)

Rogers SG, Horsch RB, Fraley RT (1986) Gene transfrer in plants: Production of transformed plants using Ti plasmid vectors. Methods Enzymol. **118:** 627-640.

Sasaki Y, Hakamada K, Suama Y, Nagano Y, Furusawa I, Matsuno R (1994) Chloroplast-encoded protein as a subunit of acetyl-CoA carboxylase in pea plant. J Biol Chem **268:** 25118-25123.

Schmidt GW, Miskind ML (1983) Rapid degradation of unassembled ribulose 1,bisphosphate carboxylase small subunits in chloroplast. Proc Natl Acad Sci USA **80:** 2632-2636.

Shintani DK, Ohlrogge JB (1995) Feedback inhibiton of fatty acid synthesis in tobacco suspension cells. The Plant Journal **7:** 577-587.

Shorrosh BS, Roesler K, Shintani DK, Van De Loo F, Savage L, Ohlrogge JB (1995) Structural analysis, plastid localization, and expression of the biotin carboxylase subunit of acetyl-CoA carboxylase from tobacco. Plant Physiology **108:** 80812.

Stitt M, Sonnewald U (1995) Regulation of metabolism in transgenic plants. Ann Rev Plant Phsyiol Plant Mol Biol **46:** 341-368.

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited nimber of gene copies may lead to a suppression of gene expression. The Plant Cell **2:** 291-299.

Chapter 6

Conclusions and Future Research Perspectives

Understanding the role of multiple ACP isoforms in plants

Acyl carrier protein (ACP) is an essential co-factor involved in fatty acid synthesis. This protein functions to shuttle the growing fatty acid chain between the enzymes of the fatty acid synthase complex. For an as yet unknown reason, many plants plastids contain multiple isoforms of ACP (Ohlrogge and Kuo, 1985). Although results from *in vitro* studies suggest that in spinach chloroplast, the different ACP isoforms may direct mature fatty acids towards different metabolic fates (Guerra, et al., 1986), presently, no *in vivo* data exist to support this hypothesis.

However, because the expression of the different plastid ACP isoforms have been extensively characterized in Arabidopsis (Hlousek-Radojcic et al., 1991; chapter 2) and DNA sequences corresponding to 4 different Arabidopsis ACP isoforms have been cloned (Lamppa and Jacks, 1991; Hlousek-Radojcic, 1991; Post-Beittenmiller et al., 1989; Chapter 2), we now have a good system to study the *in vivo* role of multiple ACP isoforms in plants. Using transgene technologies, it is now possible to alter the expression of individual ACP isoforms in Arabidopsis and determine what, if any, effect these changes would have on plant lipid metabolism.

The identification of a cDNA corresponding to the LMI ACP is particularly helpful since, unlike the previously cloned Arabidopsis ACPs, the LMI ACP is expressed almost exclusively in green tissues and is the predominant ACP isoform in leaf tissue. Arabidopsis leaf fatty acids have been shown to be partitioned between plastid and microsomal localized glycerolipid

biosynthetic pathways (Browse and Somerville, 1991). As was hypothesized by Guerra et al. (1985), LMI ACP may be involved in directing fatty acid chains towards one of these two metabolic fates. Because the plastid localized glycerolipid pathway exists primarily in photosynthetic tissues, it is logical to propose that the LMI ACP is involved in directing fatty acids towards this pathway. If this were the case, lowering the expression of the LMI ACP in green tissues might divert the flux of fatty acids away from the plastid localized glycerolipid pathway and towards the microsomal pathway. This should result in a change in the leaf fatty acid composition to resemble that of the Arabidopsis *act*1 mutant which is deficient in the ability to synthesize glycerolipids through the plastid localized pathway (Kunst et al., 1988).

Over-expression studies of the LMI ACP in a tissue where it is not normally found (i.e. the root), may also help determine what role it plays in fatty acid synthesis. LMI ACP expression in root tissue may alter the fatty acid composition of the root to more closely resemble the composition of green tissue. Similar studies are now under way where the Arabidopsis ACP-1 isoform was over-expressed in Arabidopsis leaves. ACP-1 levels have been significantly increased in leaf tissue and this isoform is now present at approximately the same level as the LMI ACP. Preliminary results from these studies suggest that the increased level of ACP-1 has increased the flux of fatty acids towards the microsomal glycerolipid pathways. Although further experimentation is needed, these results are very promising. Antisense and over-expression constructs of the LMI ACP cDNA have been constructed and are now ready to transform into Arabidopsis. These experiments are now under way and preliminary analysis of LMI antisense and over-expressing plants will be available in the near future.

An alternative approach to understanding the role of multiple plastid ACP

isoforms in plants involves the measurements of acyl-ACP pools. Post-Beittenmiller et al. (1991), reported that in spinach the two ACP isoforms participated equally in the early enzymatic steps of fatty acid synthesis. However, because they were unable to measure the long chain acyl-ACP pools of both ACP isoforms, it was not possible to make any evaluations of how the two ACPs participate in the terminal reactions of fatty acid synthesis. The purification of the LMI ACP from Arabidopsis leaves has shown that deacylated leaf ACP isoform can be fractionated by anion-exchange chromatography (Chapter 2). The resolution of ACP isoforms is in part due to the differences in the pI and hydrophobic nature of the different Arabidopsis leaf ACPs (Chapter 2). It may be possible to exploit these physical difference to fractionate the different ACPs such that we will be able to analyze acyl-ACPs of different chain lengths in an isoform specific manner. Since we have cloned four of the five different leaf ACP isoforms and expressed each in E. coli (Hlousek-Radojcic et al., 1991; Chapter 2), it is possible to synthesize acyl-ACP standards for each isoform (Kuo and Ohlrogge, 1985). These different acyl-ACP standards can then be used to establish whether acyl-ACPs can be fractionated in an isoform specific manner using different chromatographic methods. The ability to fractionate the different acyl-ACPs in an isoform specific manner will allow us to directly access their participation *in vivo* in the different terminal reactions of fatty acid synthesis.

Mitochondrial ACP and fatty acid synthesis

Cumulative evidence from mitochondrial protein import studies, sequence comparisons with known mitochondrial ACPs from animals and fungi, and immunolocalization studies have proven that the protein encoded by the

Arabidopsis mtACP-1 cDNA is targeted and localized within the plant mitochondria (Chapter 3). Furthermore, these studies indicate that the majority of mtACP-1 protein is present primarily in the acylated form and appears to be membrane associated. When the mtACP-1 was used in an *in vitro* fatty acid synthase assay, the fatty acid products were primarily medium chain fatty acids (i.e. decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0)).

I. Are plant mitochondria capable of *de novo* fatty acid synthesis and if so why?

The observations described in Chapter 3 and summarized above are consistent with those from studies of the *Neurospora* and bovine heart muscle mitochondrial ACPs. In all three cases, the acylated form of mtACP was found to be membrane associated (Runswick et al., 1991; Sackmann et al., 1991; Chapter 3). Furthermore, *Neurospora* mitochondria have been reported to be capable of *de novo* fatty acid synthesis (Brody et al., 1990; Zenson et al, 1992) which is consistent with our findings that the *Arabidopsis* mtACP-1 is able to function as a FAS co-factor.

Although through the work described in Chapter 3, we were able to prove that plants poccess a mitochondrial form of ACP, we were not able assign a function to the plant mitochondrial ACP. Our data suggesting that the *Arabidopsis* mtACP-1 participated in fatty acid synthesis was indirect and needed to be followed up with careful labelling studies with isolated mitochondria. Fortunately, Dr. Hajime Wada from the University of Kyushu in Japan, has continued the work on the plant mitochondrial ACP. Through radiolabelling studies, he has shown that pea mitochondria are capable of *de novo* fatty acid when provided with [1-^{14}C]malonate. These labelling studies

revealed that the primary fatty acid products of pea mitochondrial FAS are 1-hydroxymyristic acid and lauric acid. By analyzing the radiolabelled acyl-ACPs, Dr. Wada identified 1-hydroxymyristoyl-ACP and lauroyl-ACP as the primary mitochondrial acyl-ACP intermediates and has thus proven directly that the plant mtACP is a co-factor of mitochondrial FAS. These results also confirm the findings that, in the presence of mitochondrial ACP, medium chain fatty acids are synthesized in FAS assays (Chapter 3).

The necessity for a redundant fatty acid synthase localized in the mitochondria is curious since cellular demand for fatty acid synthesis for membrane biosynthesis appear to be met by the cytosolic and plastid localized FAS in non-photosynthetic and photosynthetic eukaryotes. It is possible that mitochondrial fatty acids are used in the post-translational acylation of specific proteins. Acylation of mitochondrial proteins has been demonstrated by feeding radiolabelled fatty acids to isolated rat liver mitochondria (Stucki et al., 1989). The mitochondrial protein acylation reaction had a substrate preference for medium chain fatty acids and radiolabelled proteins were resistant to hydrolysis by hydroxylamine and base indicating that the acylation was probably occurring through an amide-linkage. Furthermore, Vassilev et al. (1995) have reported that in *Neurospora*, cytochrome c oxidase is myristylated via an amide linkage to a specific lysine residue. These results are consistent with the findings from [1-^{14}C] malonate labelling of isolated pea mitochondria where several radiolabelled proteins were shown to be resistant to base and hydroxylamine (Wada and Ohlrogge, personal communication).

II. Is the plant mitochondrial ACP a subunit of the NADH:ubiquinone oxidoreductase (complex I)?

Groups working on NADH:ubiquinone oxidoreductase (complex I) in bovine heart and *Neurospora* mitochondria found that the acylated form of the mtACP is an integral subunit of complex I (Runswick et al., 1991; Sackmann et al., 1991). The *Neurospora* complex I has been shown to be composed of two tightly associated domains. A membrane domain, composed of proteins encoded by the mitochondrial genome, constitutes the ubiquinone hydrogenase function of complex one (Weiss et al., 1991). The other domain, which is composed primarily of proteins encode by nuclear genes, protrudes into the mitochondrial matrix and contains the NADH dehydrogenase activity (Weiss et al., 1991). The mitochondrial ACP has been shown to be associated with the matrix domain of complex I (Sackmann et al., 1991). Although it is not clear what role the mtACP plays in complex I, Schulte et al. (1994), reported that the formation of the *Neurospora* complex I is inhibited when the gene encoding the mtACP was disrupted. This observation indicates that the presence of the mtACP is an essential component of this protein complex.

The observations that only the acylated form of mtACP-1 is membrane associated and that the *Neurospora* and bovine mitochondrial ACPs are only found in the acylated form when associated with complex I suggest that the acylation may be key to mitochondrial ACPs role in complex I. It is known that protein acylation is involved in the membrane anchoring of certain proteins (McIlhinney, 1990; Towler et al., 1988). Furthermore, protein acylation has been shown to facilitate protein-protein interactions (McIlhinney, 1990; Towler et al., 1988). It is possible that the acylated form of the mitochondrial ACP is involved in the anchoring of the matrix protruding domain of complex I to either the

mitochondrial inner membrane or to the membrane domain of complex I. If this is the case, then it would be interesting to investigate the role of the plant mitochondrail ACP in complex I under conditions when electron flow is diverted away from complex I and towards the internal rotenone insensitive NAD(P)H dehydrogenase in plants. It is possible that the two domains of complex I could become uncoupled through the deacylation of the mtACP-1. Such a mechanism would inactivate complex I and allow NADH reduction to occur through the rotenone insensitive NAD(P)H dehydrogenase.

So far there is little direct evidence for the involvement of the mitochondrial ACP in the NADH:ubiquinone oxidoreductase complex in plants. We have recently sent antibodies to the *Arabidopsis* mtACP to a group studying the *Arabidopsis* NADH:ubiquinone oxidoreductase. Hopefully, they will be able to determine if mtACP-1 is present in complex I. Additionally, Dr. Wada is attempting to alter the expression of the mtACP-1 in *Arabidopsis* using antisense approaches and he is now in the process of analyzing plants transformed with the mtACP-1 antisense construct. Although a total knock out of mtACP-1 expression is likely to be lethal to the plant, it is possible that decreased expression of mtACP-1 will lead to a decrease in mitochondrial respiration due to decreased capacity for electron transfer from complex I.

III. Multiple isoforms of ACP may be present in Arabidopsis mitochondria.

Recently, we have identified a cDNA clone from the *Arabidopsis* EST cDNA collection that encodes a protein that shows a high degree of sequence similarity to the *Arabidopsis* mtACP-1 protein and other known mitochondrial ACP sequences. Although similar to mtACP-1, the newly identified cDNA clone appears to correspond to an unique mitochondrial ACP isoform. We are now in

the process of characterizing this cDNA clone. This finding suggest, that like plastids, mitochondria may also contain multiple isoforms of ACP.

Product feedback regulation of plant fatty acid synthesis

Plant fatty acid synthesis is tightly regulated such that fatty acids are produce only at quantities needed to meet the demand of glycerolipid biosynthetic enzymes. The results presented in Chapter 4 indicate that plants employ a product feedback mechanism to control the flux through the fatty acid biosynthetic pathway. In these studies, tobacco suspension cultures treated with exogenous fatty acid in the form of Tween-18:1 incorporated [1-^{14}C]acetate into fatty acids at a rate 3 to 5 fold slower than untreated cells. Analysis of acyl-ACP intermediate pools isolated from Tween treated and control cells revealed that fatty acid synthesis was being regulated by feedback inhibition occurring at the level of acetyl-CoA carboxylase (ACCase). These studies also showed that the long chain acyl-ACPs were rapidly turned over upon the addition of Tween-18:1 indicating that the long chain acyl-ACP thioesterases were not affected by the feedback regulation. The feedback inhibition appeared to be occurring through a biochemical mechanism since ACCase subunits and fatty acid synthase enzyme levels were not altered in response to the feedback inhibition. Preliminary studies have shown that the feedback inhibition of fatty acid synthesis in tobacco suspension cells is rapidly reversed by the removal of Tween-18:1. The rapid onset and reversal of the inhibition are consistant with control occuring at the biochemical level.

I. What is the feedback molecule involved in the feedback inhibition of plant fatty acid synthesis?

Although ACCase was identified as an important regulatory enzyme involved in the feedback inhibition of fatty acid synthesis, the biochemical mechanism by which the inhibition occurs is unknown. The classical feedback inhibition mechanism involves the allosteric modification of the regulatory enzyme caused by the binding of a product or particular pathway intermediate. It is possible that such a mechanism is employed in plants during feedback inhibition of fatty acid synthesis, however at this point, the identity of the feedback molecule is not known.

Several potential candidates exist for the feedback molecule involved in the inhibition of plant fatty acid synthesis. Long chain acyl-ACPs have been suggested as likely feedback molecules. In *E. coli*, the depletion of long chain acyl-ACPs caused increases in the rate of ACCase and fatty acid synthesis (Ohlrogge et al., 1995; Jiang and Cronan; 1994). These results suggest that long chain acyl-ACPs act as feedback molecules involved in the product inhibition of E. coli fatty acid synthesis. However, long chain acyl-ACPs do not seem to play the same role in plants. The observations that the long chain acyl-ACP pools are rapidly turned-over upon initiation of feedback inhibition of plant fatty acid synthesis indicates that these FAS intermediates are probably not involved in in the feedback mechanism. Furthermore, long chain acyl-ACPs had little effect on ACCase activity when added to *in vitro* ACCase assays (Roesler et al., 1996). It is also not likely that the medium chain acyl-ACPs are functioning as feedback molecules since the sizes of these acyl-ACP pools remained constant under both control and feedback conditions.

The results from *in vitro* ACCase assays also cast doubt on the role of free

fatty acids as feedback molecules since the addition of palmitic (16:0) and oleic (18:1) acids did not inhibit ACCase activity (Roesler et al., 1995).

Another possible inhibitor molecule that may interact directly with ACCase is acetyl-ACP. Acetyl-ACP levels rapidly increase upon treatment with Tween-18:1. The accumulation of acetyl-ACP closely correlated with the degree of feedback inhibition observed in Tween treated cells. It is possible that the initial inhibition of FAS is occurring at B-ketoacyl-ACP synthase I (KAS I), the enzyme responsible for the turn-over of the acetyl-ACP pool. This inhibition could result in a subsequent increase in acetyl-ACP which results in the inhibition of ACCase. This hypothesis would require acetyl-ACP to be a highly potent inhibitor of ACCase such that the Ki for acetyl-ACP would be very low. However, in dispute of this hypothesis, Jaworski et al. (1993) suggest that the accumulation of acetyl-ACP is a direct consequence of decreased ACCase activity. They propose that acetyl-ACP levels increase because, due to decreased availability of malonyl-CoA, acetyl-ACP can not be utilized in condensation reactions catalyzed by B-ketoacyl-ACP synthase I. To distinguish between these alternatives, we could test the effect of acetyl-ACP on ACCase ativtiy in *in vitro* assays. If ACCase proves to be inhibited by acetyl-ACP, studies should then be done look at the effect of potential feedback molecules (i.e. free fatty acids and acyl-ACPs) on KAS I. If this hypothesis is correct, then it would appear that the inhibition of ACCase would be a secondary effect of the primary inhibition occurring at a different point in plant fatty acid synthase.

II. Interaction between plastid fatty acid synthesis and microsomal glycerolipid synthesis.

Rates of plastid fatty acid synthesis must be coordinately regulated with

cytosolic demands for fatty acids. Therefore, the feedback of fatty acid synthesis must involve inter-organellar communication between these two pathways. The link between these two pathway appears to involve the cytosolic acyl-CoA pools. The cytosolic acyl-CoAs are synthesized in the chloroplast outer envelope membrane. After being cleaved from ACP backbones by acyl-ACP thioesterases, free fatty acids move across the chloroplast envelope where they are ligated to coenzyme A (CoA) in a reaction catalyzed by acyl-CoA synthetase. These acyl-CoAs serve as substrates for microsomal glycerolipid synthesis. The cytosolic acyl-CoA pools are very small and difficult to measure. It is likely that plant cells try to maintain a low concentration of long chain acyl-CoAs because due to their detergent nature, accumulation of these compounds may be toxic. The addition of exogenous fatty acids is likely to over-load the cytosolic acyl-CoA pools. The plant cell may then sense this increase in cytosolic acyl-CoA and act to prevent the *de novo* synthesis of fatty acids. Studies suggest that although feedback inhibition of fatty acid synthesis occurred upon the addition of exogenous fatty acids, there was little direct interaction between the exogenously applied fatty acid and the plastid localized fatty acid biosynthetic enzymes. Terzaghi (1986a & b) determined that rabiolabelled oleic acid (18:1) derived form Tween-18:1 incorporated almost exclusively into microsomal glycerolipid biosynthetic pathways and little or no label was present in plastid derived glycerolipids.

One possible point of regulation is at the plastid outer envelope acyl-CoA synthetase. Studies indicate that acyl-CoA synthetase is inhibited by the accumulation of acyl-CoA. In preliminary expreriments, acyl-CoA synthetase activity was inhibited by the addition of oleoyl-CoA (18:1-CoA) or palmitoyl-CoA (16:0-CoA) (data not shown). These results are consistant with the hypothesis that a primary cytosolic factor, possibly long chain acyl-CoAs, may

be involved in sensing the need to decrease rates of fatty acid synthesis.

To test the hypothesis that cytosolic factors are involved in communicating the demand of microsomal glycerolipid pathways for increases or decreases in fatty acid synthesis, both biochemical and molecular approaches can be applied. The major thrust of these studies should focus on the role of the cytosolic acyl-CoA pools in communication between the plastid and microsomal pathways. Initial studies could be done to see if the acyl-CoA or free CoA pools are altered by the addition of exogenous fatty acids. Acyl-CoAs could be analyzed from tobacco suspension cells treated with different levels of Tween-18:1 to determine the capacity of the acyl-CoA pools to accept exogenous fatty acids. These studies could be conducted in conjunction with [1-^{14}C]acetate labelling studies to determine if there is a relationship between rates of fatty acid synthesis and changes in the acyl-CoA pools.

Transgenic plants offer another approach to examine thet effect of perturbing the cytosolic acyl-CoA pool on inter-organellar communication. Several groups (i.e. Ernst Heinz, University of Hamburg; Matthew Hills, John Innes) have been started to characterize plant acyl-CoA synthetases and several acyl-CoA synthetases have recently been identified from the Arabidopsis EST cDNA collection. These efforts will result in the identification of a cDNA clone corresponding to a plastid outer envelope localized acyl-CoA synthetase. Once such a clone is in hand, it will be possible to determine if under- and over-expression of this acyl-CoA synthetase in transgenic plants. Once plants with altered acyl-CoA synthetase levels have been engineered, one can determine what effect these changes will have on the plastid fatty acid synthesis and the microsomal glycerolipid pathways' ability to respond to the addition of exogenous fatty acids. These studies may lead to a an understanding of the

cytosolic acyl-CoA pool role in regulating interorganellar lipid metabolism.

Antisense- and over-expression of the biotin carboxylase gene in transgenic tobacco leaves.

As was described in chapter 5, initial experiments were conducted to evaluate the effect of altered biotin carboxylase gene expression on the plastid acetyl-CoA carboxylase (ACCase) complex formation and leaf fatty acid metabolism in transgenic tobacco. Biotin carboxylase is a subunit of the heteromeric plastid localized ACCase. Biotin carboxylase and three other protein subunits, specifically biotin carboxyl carrier protein (BCCP), and the alpha and beta subunit of carboxyltransferase, combine to form the plastid ACCase complex. ACCase catalyzes the first committed step of fatty acid synthesis in which acetyl-CoA is carboxylated to malonyl-CoA. The results from several *in vivo* studies indicate that the plastid ACCase plays a role in regulating rates of plant fatty acid synthesis. Tobacco plants were transformed with constructs containing the tobacco biotin carboxylase gene expressed in the sense and antisense orientation behind the caulifower mosaic virus 35S transcript (CaMV) promoter. Plants generated from these transformations contained leaf biotin carboxylase expression levels ranging from 10% to 1000% of wild type leaf levels. Altered levels of leaf biotin carboxylase had no effect on BCCP levels indicating that the subunits of the plastid ACCase were not maintained in a strict stoichiometric ratio. Although the phenotype and leaf fatty acid content of plants showing high and moderately low levels of biotin carboxylase were indistinguishable from wild type plants, stunted growth and decreased leaf lipid content was observed in a single tranformant, A11, that was severely deficient in leaf biotin carboxylase. The altered phenotype and leaf fatty acid content was

only observed when the A11 plant was grown under low light conditions.

I. Is the stunted phenotype and decreased leaf fatty acid content of A11 plants grown under low light conditions due to decreased biotin carboxylase levels?

Under low light conditions the severe decrease in biotin carboxylase levels in the leaves of A11 plants was associated with a decrease in leaf fatty acid content and stunted phenotype. These effects could be attributed to decreased ACCase levels, which subsequently caused the plant to be unable to synthesized fatty acids at rates sufficient to maintain normal growth. Alternatively, it is possible that the p0719A T-DNA inserted aberrantly into the tobaco genome which resulted in the A11 phenotype. In order to distinguish between these two possibilies, a series of diagnostic genetic crosses were made with homozygous A11 plants.

The A11 phenotype may be due to an insertion of the p0719A T-DNA into the tobacco genome which resulted the inactivation of an essential gene. Such a mutation would most likely be recessive in nature. To determine if this were the case, a homozygous A11 plant was backcrossed with wild type tobacco. The progeny of this cross will be heterozygous for the T-DNA insertion and the wild type locus. If the stunted A11 phenotype is the result of a recesive mutation caused by the insertion of the T-DNA, one would expect the progeny to no longer express the A11 phenotype due to the complementation by the wild type locus. However, if the phenotype was due to the decrease in biotin carboxylase, the progeny should appear stunted. The progeny of the back cross have been germinated on kanamycin and were recently transferred to soil. So far, stunted growth has not been detected in these plants.

One other possible explanation for the A11 phenotype is that the p0719A T-DNA inserted into a regulatory region of some unknown gene causing a dominant phenotype. To address this possiblity we crossed the homozygous A11 plant with transgenic tobacco expressing a cytosolic ACCase gene which has been engineered to contain a chloroplast transit peptide. Unlike the heteromeric plastid ACCase, the cytosolic ACCase is a homodimer of a single multifunctional polypeptide that contains all of the ACCase subunits. When this engineered cytosolic ACCase construct was transformed into *Brassica napus*, it was correctly targeted to the plastid in an active form (Ohlrogge, personal communication). If the A11 phenotype was due to the decrease in biotin carboxylase which resulted in decreased ACCase activity, the cross with the tobacco plant containing the plastid targeted homomeric ACCase should result in progeny that no longer exhibit the A11 phenotype. We are now in the process of analyzing the progeny from this cross. So far these plants are not exhibiting the stunted phenotype that is associated with the A11 plant. Further analysis of these plants will need to be done to confirm this preliminary result.

In further support for the hypothesis that the stunted phenotype was due to the antisense expression of biotin carboxylase, the growth of some of the progeny from the A14 self cross were also stunted when grown under low light conditions. The original A14 transformant contained approximately 50% of wild type biotin carboxylase levels in its leaves and grew normally under both low light and greenhouse conditions. It is possible that the stunted phenotype observed in the progeny of the A14 self cross is due to co-segragation of multiple copies of the biotin carboxylase antisense loci. Again as in all of the diagnostic crosses described above further analysis needs to be done on these plants to confirm the conclusions made from these preliminary observations.

II. Why is stunting observed in plants expressing low levels of biotin carboxylase only under low light conditions?

If the A11 phenotype proves to be due to the observed decrease in biotin carboxylase levels, then we need to understand why the phenotype is only exhibited when plants are grown under low light conditions. Initial experiments can be done to determine at what light intensity do biotin carboxylase levels become limiting. We can dertermine this in two ways. First, A11 plants can be grown under different light intensities and patterns of growth can be recorded. Second, [1-*14*C]acetate labelling experiments can be done under different light intensities on leaf disc isolated from A11 and wild type plants. The leaf disc will be taken from A11 and wild type plants that are grown under high light conditions to ensure that the tissues are developmentally similar. By comparing rates of label incorporation into fatty acids, we can determine at what light intensity biotin carboxylase becomes limiting. The next set of experiments will set out to answer how the limitation in biotin carboxylase effects ACCase activity. We will attempt to isolate intact ACCase complexes from leaves of A11 plants grown under various light intensities and determine if the subunit stoichiometry of the ACCase complex is altered in low light relative to high light grown plants. Because antibodies to each of the ACCase subunits are available we should be able to access levels of each subunit. However, we need to develop methods to isolate active tobacco chloroplast ACCase complexes. This has so far proven difficult and is possibly due the the high level of phenolics in tobacco leaves. New methods of tobacco chloroplast isolation may be necessary to address this question.

Literature Cited

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

Guerra DJ, Ohlrogge JB, Frentzen M (1986) Activity of acyl carrier protein isoforms in reactions of plant fatty acid metabolism. Plant Physiol **82:** 448-453.

Hlousek-Radojcic A, Post-Beittenmiller D, Ohlrogge JB (1991) Expression of constitutive and tissue specific acyl carrier protein isoforms in *Arabidopsis*. Plant Physiol **98:** 206-214.

Jaworski JG, Post-Beittenmiller D, Ohlrogge JB (1993) Acetyl-acyl carrier protein is not a major intermediate in fatty acid biosynthesis in spinach. FEBS Lett **213:** 981-987.

Jiang P, Cronan JE (1994) Inhibition of fatty acid synthesis in Escherichia coli in the abscence of phospholipid synthesis and release of inhibition by thioesterase action. J Bacteriol **176:** 2814-2821.

Kuo TM, Ohlrogge JB (1984) Acylation of plant acyl carrier proteins by acyl acyl carrier protein synthetase from *Escherichia coli*. Arch Biochem Biophys **230:** 110-116.

Kunst L, Browse J, Somerville C (1988) Altered regulation of lipid biosynthesis in a mutant of *Arabidopsis* deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc Natl Acad Sci USA **85:** 4143-4147.

Lamppa G, Jacks C (1991) Analysis of two linked genes coding for the acyl carrier protein (ACP) from *Arabidopsis thaliana*. Plant Mol Bio **16:** 469-474.

McIlinney RAJ (1990) The fats of life: the importance and function of protein acylation. TIBS **15:** 387-390.

Ohlrogge JB, Savage L, Jaworski J, Voelker T, Post-Beittenmiller D (1995) Alteration of acyl-acyl carrier protein pools and acetyl-CoA carboxylase expression in *Escherichia coli* by a plant medium chain acyl-acyl carrier protein thioesterase. Arch Biochem Biophys **317:** 185-190.

Ohlrogge JB, Kuo TM (1984) Plants have isoforms for acyl carrier protein that are expressed differently in different tissues. J Biol Chem **260:** 8032-8037.

Post-Beitenmiller MA, Hlousek-Radojcic A, Ohlrogge JB (1989) DNA sequence of a genomic clone encoding an *Arabidopsis* acyl carrier protein (ACP). Nucleic Acids Res. **17:** 1777.

Post-Beittenmiller MA, Jaworski JG, Ohlrogge JB (1991) In vivo pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem **266:** 1858-1865.

Roesler KR, Savage LJ, Shintani DK, Shorrosh BS, Ohlrogge JB (1995) Co-purification, co-immunoprecipitation, and coordinate expression of acetyl-CoA carboxylase activity, biotin carboxylase, biotin carboxyl carrier protein of higher plants. Planta **198:** (in press)

Runswick MJ, Fearnley IM, Skehel JM, Walker JE (1991) Presence of an acyl carrier protein in NADH:ubiquinone oxidoreductase from bovine heart mitochondria. FEBS Lett **286:** 121-124.

Sackmann U, Zenson R, Rohlen D, Jahnke U, Weiss H (1991) The acyl carrier protein in *Neurospora crassa* is a subunit of NADH:ubiquinone reductase (complex I). Eur J Biochem **200:** 463-469.

Schulte U Fecke W, Krull C, Nehls U, Schmiede A, Schneider R, Ohnishi T, Weiss H (1994) In vivo dissection of the mitochondrial respiratory NADH:ubiquinone oxidoreductase (complex I). Biochim Biophys Acta **1187:** 121-124.

Stucki JW, Lehmann LH, Siegel E (1989) Acylation of proteins by myristic acid in isolated mitochondria. J Biol Chem **264:** 6376-6380.

Terzaghi WB (1986a) A system for manipulating the membrane fatty acid composition of soybean cell cultures by adding Tween fatty acid ester to their growth medium. Plant Physiol **82:** 771-779

Terzaghi WB (1986b) Metabolism of Tween-fatty acid esters by cultured soybean cells. Plant Physiol **82:** 780-786.

Towler DA, Gordon JI, Adams SP, Glaser L (1988) The biology and enzymology of eukaryotic protein acylation. Ann Rev Biochem **57:** 69-99.

Vassilev AO, Plesofsky-Vig N, Brambl R (1995) Cytochrome c oxidase in Neurospora crassa contains myristic acids linked to subunit 1. Proc Natl Acad Sci USA **92:** 8680-8684.

Weiss H, Frieddrich T, Hofhaus G, Preis D (1991) The repiratory-chain NADH dehydrogenase (complex I) of mitochondria. Eur J Bichem **197:** 563-576.