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presented by

Gustavo Bonaventure

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UNDERSTANDING GENE EXPRESSION AND METABOLIC CONTROLS ON FATTY ACID BIOSYNTHESIS IN PLANTS

By

Gustavo Bonaventure

A DISSERTATION

Submitted to
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ABSTRACT

UNDERSTANDING GENE EXPRESSION AND METABOLIC CONTROLS ON FATTY ACID BIOSYNTHESIS IN PLANTS.

By

Gustavo Bonaventure

Plant fatty acids represent an important renewable source of highly reduced carbon chains that can be used for purposes ranging from food production to industrial feedstocks. So far, many attempts to engineer fatty acid production in plants have been based on current knowledge of the fatty acid metabolic pathway and the structural genes involved in it. However, plant tissues have proven to be not easily amenable to fatty acid engineering by overexpression of single FAS enzymes. These experiments lead to the conclusion that identification of regulatory factors limiting fatty acid production in plant tissues is crucial for manipulation of this metabolic pathway. At present, these factors have not been identified. Therefore, one major goal of this study has been to understand the signals and mechanisms that control fatty acid biosynthesis and the expression of genes for this primary metabolic pathway in plants.

The results presented in this study demonstrate that acyl carrier protein (ACP) genes in Arabidopsis are under multiple levels of controls and suggest that some signals known to activate fatty acid gene expression in non-plant organisms have been conserved in plants (e.g., transcriptional activation by growth/cell cycle) whereas others differ (e.g., transcriptional activation by feedback mechanisms). Light has an important role on expression of ACP genes, increasing ACP4 mRNA levels and promoting the association of ACP transcripts with polyribosomes. Thus, this study demonstrates that different ACP

genes are regulated by specific signals depending on the tissue and its fatty acid requirements.

In addition, this thesis has explored the production of saturated fatty acids and their partitioning between diverse cellular processes by focusing on the characterization of an Arabidopsis acyl-ACP thioesterase mutant (*fatb-ko).* Analysis of this mutant has revealed the central role of FATB in the production of saturated fatty acids in all tissues. Moreover, redistribution of these molecules between different biosynthetic pathways in *fatb-ko* suggests that cells prioritize the synthesis of critical components for growth. In addition, slower growth and production of non-viable seeds in the mutant demonstrate an essential role of saturated fatty acids in plant growth and seed development.

Isotope labeling experiments and western blots were used to investigate fatty acid metabolism and protein expression in *fatb-ko* leaves. The results indicate that *fatb-ko* leaves increase the rate of fatty acid synthesis by 40 % compared to wild type. The mutant also increases by 70% the initial rate of fatty acid breakdown. Western blot analysis reveal that BCCP, ACPs and 18:0-ACP desaturase levels are increased by 1.5- to 2-fold in mutant leaves compared to wild type. Thus, plant cells appear to have mechanisms that sense and respond to subnormal levels of saturated fatty acids in the cytoplasm. These mechanisms probably coordinate the requirements for lipid synthesis in the cytosol and fatty acid production inside the plastids. Moreover, the same mechanisms may also activate expression of nuclear genes for fatty acid synthesis.

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CHAPTER 1

INTRODUCTION

"It takes a membrane to make sense out of disorder in biology. When the earth came alive, it began constructing its own membrane, for the general purpose of editing the sun. You have to be able to catch energy and hold it, storing precisely the needed amount and releasing it in measured shares. A cell does this, and so do the organelles inside. Each assemblage is poised in the flow of solar energy, tapping off energy from metabolic surrogates of the sun. To stay alive, you have to be able to hold out against equilibrium, maintain imbalance, bank against entropy, and you can only transact this business with membranes in our kind of world", **Lewis Thomas**.

———————— o ————————

Plant lipids are extremely diverse in structure and function (Table 1) and constitute the products of several distinct biosynthetic pathways. These molecules serve several essential functions in all organisms but their most critical role for life is the formation of cellular membranes. These membrane structures form the major barriers that delineate cells and their compartments and allow essential processes to occur (e.g., anabolic and catabolic reactions, transport by vesiculation and permeation, photon capture) (Rausch and Bucher, 2002; Bowsher and Tobin, 2001; Nebenfuhr and Staehelin, 2001).

Among the many types of plant lipids, glycerolipids are usually the most abundant in plant cells (Ohlrogge and Browse, 1995). These lipids are the main constituent of cellular membranes and are derived from the glycerol and fatty acid biosynthetic pathways (Somerville *et al.*, 2000). The fatty acid synthesis (FAS) pathway is a primary metabolic pathway, because it is found in every cell of the plant and is essential for growth. In addition to supplying fatty acids for the synthesis of membrane lipids, this pathway provides substrates for several other essential cellular processes in plants. For example,

synthesis of sphingolipids, epicuticular waxes and cutin in epidermal cells and triacylglycerols in seeds. Moreover fatty acids are substrates for acylation reactions (e.g., proteins, sterols) and also precursors of signaling molecules (Figure 1).

Table 1. Functions of lipid molecules in higher plants

Function	**Lipid types**
Membrane components	Glycerolipids, Sphingolipids, Sterols
Storage compounds	Triacylglycerols, Waxes
Compounds active in electron transfer	Chlorophylls, Ubiquinone, Plastoquinone
Photoprotection and free radical protection	Carotenoids, Tocopherols
Surface protection	Cutin, Suberin, Waxes, Triterpenes
Protein modification	14:0, 16:0, Farnesyl and GeranylGeranyl pyrophosphate, Phosphatidylinositol, Ceramides, Dolichol
Signaling	Jasmonate, Diacylglycerol, ABA, GA, Brassinosteroids

As a result of the dependence of multiple cellular processes on the production of fatty acids, the synthesis of these molecules is exquisitely controlled to balance supply and demand for acyl chains. For plant cells, this means matching the level of fatty acid synthesis to membrane biogenesis and to multiple cellular pathways depending on the

Figure 1. Fatty acids exported from plastids have diverse roles in plant cells.

In mesophyll cells fatty acids are used primarily for membrane glycerolipid synthesis. In epidermal cells the bulk of fatty acids goes into waxes and cutin. In seeds, triacylglycerols accumulate most of the exported fatty acids. In most cells saturated fatty acids are precursors of ceramides and substrates of acylation reactions.

Cytosol
Plastid
Fatty Acid
Biosynthesis
16:0
18:0
18:1
saturated
fatty acid
unsaturated
fatty acid
membrane
glycerolipids
waxes
cutin
Cell
wall
wax, cutin
(epidermis)
glycerol
fatty
acids
TAG
(seeds)
Protein
Ceramide and
sphingolipids

Figure 1

tissue, growth rate, stage of development and time of the day. For example, leaves increase by more than 10-fold the production of fatty acids during the day and some seeds accumulate 60 % of their weight in oil (Ohlrogge and Jaworski, 1997). Thus, a critical biochemical aspect of fatty acid biosynthesis in plants is to understand how cells adjust the production of fatty acids according to different demands and how they partition these molecules into several cellular pathways. Completion of the Arabidopsis genome sequence has accelerated the identification of structural enzymes involved in the synthesis of fatty acids and glycerolipids, and most of them have been either experimentally described or have strong candidates (Beisson *et al.*, 2003; Mekhedov *et al.*, 2000). By contrast, advances in the identification of regulatory factors and mechanisms of control for this metabolic pathway have been slower. Thus, a number of major question in the synthesis of fatty acids in plants remain unanswered:

- How are fatty acid and lipid biosynthetic genes regulated?
- Do global *trans*-acting factors exist that simultaneously control the expression of most genes?
- What signals control the promoter activity of these genes?
- How is the production of fatty acids controlled in plants?
- What signals are sensed to adjust fatty acid synthesis rates to different cellular demands of fatty acids?
- How is the production of fatty acids in the plastids coordinated with their utilization outside this organelle?

- Do signals communicate between the cytosol and the plastid fatty acid synthesis pathway?

Objectives

Based on these questions, the main objectives of the present work are to understand the signals that control the rate of fatty acid synthesis in plants, the expression of genes for this primary pathway and the production and partition of exported fatty acids from plastids.

Understanding regulation of FAS genes in Arabidopsis

To begin to answer some of these important aspects of plant gene regulation and metabolism, Chapter 2 presents data on the signals and mechanisms that regulate the expression of Arabidopsis ACP isoforms. Why do we study ACP gene expression? First, these proteins are in the core of the fatty acids synthesis machinery and play a central role during acyl chain synthesis. Due to this pivotal role, it is likely that signals and mechanisms that affect ACP expression will also affect the expression of other fatty acid genes. Second, ACP mRNA and protein levels are among the most abundant of the fatty acid pathway, which in general are of low abundance. Third, ACP expression studies such as tissue specific expression and promoter analysis have been previously reported and provide partial but important information about regulatory signals and mechanisms

(Baerson *et al.*, 1994; Hlousek-Radojcic *et al.*, 1992; Battey and Ohlrogge, 1990; Hannapel and Ohlrogge, 1988).

Transcriptional regulation of genes for fatty acid synthesis

Clues to the signals and mechanisms that activate plant FAS gene expression can be obtained from known signals and mechanisms in non-plant organisms, if some of these signals and mechanisms are conserved between distantly related living organisms. Considering the universal role of lipids in cell structure and regulatory processes it may be possible that fatty acid production and its genetic regulation show, in some aspects, close resemblance in divergent organisms. For the purpose of reviewing how FAS genes are controlled in non-plant organisms, a short overview of transcriptional regulation of FAS genes in these organisms is given below. In addition, a brief summary of previous studies that analyzed different aspects of FAS gene expression in plants is presented.

Transcriptional regulation of FAS genes in microorganisms

The genes for fatty acid metabolism in *E. coli* are scattered about the genome with only two clusters, the minimal *accBC* and the *fab* cluster (Cronan and Rock, 1996). All genes are under regulation of FadR, a protein with dual functions that represses genes involved in fatty acid degradation and activates genes involved in fatty acid synthesis. In the absence of long chain acyl-CoA, FadR directly binds to specific DNA sequences to simultaneously activate transcription of biosynthetic genes and repress catabolic genes.

Thus, during exponential growth or in the absence of exogenous fatty acids, the pool of acyl-CoA remains very small and FadR activates biosynthetic genes and shuts off catabolic gene expression. When growth slows down or exogenous fatty acids are abundant, the acyl-CoA levels build up and FadR de-activates expression of FAS genes and de-represses catabolic genes. Thus, in these organisms, FAS genes are almost exclusively controlled by growth or exogenous fatty acids and respond to the demand for new membranes (Cronan and Rock, 1996).

In *B. subtilis* the genes involved in fatty acid metabolism are located mainly in gene clusters (e.g., the *fabHAF* operon) and are coordinately regulated by FapR. This regulator is a transcription factor that represses the expression of lipid metabolic genes by binding to a consensus sequence contained in their promoter regions (Schujman *et al.*, 2003). Thus, expression of fatty acid gene clusters is de-repressed when cells are in the exponential phase of growth. Interestingly enough, addition of inhibitors of fatty acid biosynthesis (e.g., cerulenin) can also de-repress these genes, suggesting that feedback mechanisms of gene expression exist in this organism. During inhibition of FAS by cerulenin, the intracellular concentration of malonyl-CoA is increased (Heath and Rock, 1995) and it is proposed that intracellular levels of malonyl-CoA regulates FapR activity. Thus, if the rate of fatty acid synthesis falls below the normal levels (as a result of slow growth or inhibition), a transient increase in the intracellular concentration of malonyl-CoA relieves FapR mediated repression of lipid biosynthetic genes (Schujman *et al.*, 2003).

In yeast, the synthesis of fatty acids is catalyzed by two multifunctional enzymes, acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS). ACC is a tetramer of identical subunits encoded by the *FAS3* gene (Schuller *et al.*, 1992). FAS consists of two multifunctional proteins, α and β, which are encoded by two unlinked genes, *FAS1* and *FAS2*, respectively (Chirala, 1992). *FAS1, FAS2* and *FAS3* genes are coordinately regulated by growth and exogenous fatty acids. Moreover, when FAS2 is over-expressed, expression of both FAS1 and FAS3 is also increased, suggesting that the FAS genes are coordinately regulated in yeast. The conserved GCCAAA element (UAS_{fas}) is present in all three *FAS* promoters and specifically enhances the transcription of *FAS* genes. In addition, *FAS1* and *FAS2* share an UAS_{ino} element that is common to genes involved in phospholipid biosynthesis. UAS_{ino} is a positive regulator of gene expression and is required for efficient expression of these genes. UAS_{fas} and UAS_{ino} act synergistically for optimal expression of the *FAS* genes (Chirala, 1992; Schuller *et al.*, 1992). In summary, microorganisms possess global *trans*-acting factors that bind to consensus sequences conserved in most of their FAS gene promoters and coordinately control the expression of most of these genes.

Transcriptional regulation of FAS genes in animals

Animals obtain fatty acids almost exclusively from their diet, and they have developed mechanisms that sense lipidic molecules such as cholesterol, fatty acids, fat-soluble vitamins and other lipids present in their food. These molecules or their derivatives bind

to receptors in the nucleus or cytoplasm that directly or indirectly activate gene expression (Chawla *et al.*, 2001).

For example, one important group of receptors is the PPAR family (peroxisome proliferator-activated receptor), that is activated by polyunsaturated fatty acids and eicosanoids. PPARs belong to the nuclear receptor superfamily of transcription factors that contain a large COOH-terminal region with a ligand binding domain. Upon ligand binding, these transcription factors form heterodimers with the retinoid X receptor (RXR) and bind to specific DNA sequences found in their target promoters known as hormone response elements (HRE) (Chawla *et al.*, 2001). Among their functions, PPARs can up-regulate proteins that increase metabolism and transport of fatty acids into the peroxisome and promote fat storage in the liver (Chawla *et al.*, 2001).

A second group of regulatory factors of fatty acid metabolism in animals is the sterol regulatory element binding protein (SREBP) family. These transcription factors are located on ER membranes in an inactive form, and are released by proteolysis in order to enter the nucleus. Proteolysis of SREBPs is inhibited by sterols and polyunsaturated fatty acids in mammals, providing a feedback regulatory mechanism for lipid synthesis (Dobrosotskaya *et al.*, 2002). SREBPs interact with an escort protein, SCAP (SREBP cleavage-activating protein) that serves as sensors of sterols, fatty acids or phospholipids in animal cells (Seegmiller *et al.*, 2002).

Transcriptional regulation of FAS genes in plants

Similarly to other higher eukaryotes, multiple *cis* elements and *trans* factors are expected to be involved in regulation of plant fatty acid gene expression. However, in contrast to animals, which obtain fatty acids mainly from their diets, plants depend on their own production of fatty acids to subsist. In addition, the plant fatty acid machinery resembles that from bacteria and is plastid localized, although almost entirely nuclear encoded. Thus, regulatory mechanisms of fatty acid gene expression that communicate between plastids and nucleus are probably present in plant cells (Jarvis, 2001). The essential differences in the structure of fatty acid synthesis between animals and plants are most likely reflected in the mechanisms and signals by which fatty acid and lipid genes are regulated in these organisms. For example, no obvious homologues of animal PPARs and SREBPs are evident in the Arabidopsis genome.

One group of plant fatty acid genes that has received special attention is the ACP gene group. Baerson and Lamppa (1993) fused an approximate 900 bp fragment upstream of the Arabidopsis *ACP2* gene to β-glucuronidase (GUS) and used the construct to generate transgenic tobacco plants. GUS expression was detected during seed development, in young leaves, in leaf epidermis, and flowers. In a second study, the same authors analyzed a series of six deletions of the same promoter in tobacco transgenic plants (Baerson *et al.*, 1994). The results revealed distinct regions of the promoter involved in vegetative and reproductive development. A –320 to –236 bp region was important for expression of the reporter gene in leaves, whereas it did not alter expression in seeds and flowers. Seed expression was reduced when a –235 to –55 bp region was deleted. This

region was also essential for expression in flowers. Thus, transcriptional regulation of the Arabidopsis *ACP2* gene in different tissues involves different regions of the promoter and presumably tissue specific factors.

Similarly, a deletion analysis of the Arabidopsis enoyl-ACP reductase promoter showed that three domains of the promoter were important for differential tissue expression. First, seed expression was unchanged by deletion to –47 bp of the transcription start site, suggesting that seed specific *cis* elements are located close to the transcription starting site. Second, removal of an intron in the 5'UTR resulted in increased expression in roots, suggesting the presence of negative regulatory elements in this region. A third region was important for high expression in young leaf tissue.

Other studies reported the light-dependent expression of *FAD7* (plastidial desaturase) (Nishiuchi *et al.*, 1995), cold-dependent expression of *FAD8* (also a plastidial desaturase) (Vijayan and Browse, 2002), and low-phosphate induction of *SQD1* and *SQD2* (Yu *et al.*, 2002; Essigmann *et al.*, 1998). Thus, a subset of genes involved in lipid metabolisms respond to specific signals depending on their distinct functions. In addition to specific environmental signals, the fatty acid and lipid genes that have been analyzed so far show a common denominator, high levels of expression in rapidly expanding tissue where cell division is active (e.g., apical meristems), and also in developing seeds and flowers, where lipid accumulation occurs.

Summary of Chapter 2

In Chapter 2, we tested signals known to activate fatty acid and lipid gene expression in non-plant organisms for their ability to activate fatty acid genes in plant cells (e.g., growth-dependent expression, inhibition of fatty acid synthesis, sugar-sensing mechanisms). Results suggested that some of these signals but not all differentially increase expression of some ACP isoforms. Thus, it appears that some regulatory signals have been conserved throughout evolution between plants, animals and lower organisms. For example, similarly to bacteria and yeast, ACP2 expression appears to respond to growth/cell division signals, whereas inhibition of fatty acid synthesis has no effect on gene expression linked to fatty acid metabolism in plants. Analysis of the Arabidopsis ACP2 promoter demonstrated that activation by growth signals acts at the transcriptional level and identified promoter regions important for regulation and expression of ACP2. Light, which stimulates growth and chloroplast development has an important role on ACP gene expression at the levels of ACP4 mRNA abundance and association of ACP transcripts with polysomes. We also demonstrated that the 5'UTR of ACP transcripts increases gene expression and determines tissue specific expression. In summary, Chapter 2 discloses multiple levels of control on ACP gene expression and provides the basis for further exploration of gene regulation for fatty acid metabolism and also identification of regulatory factors for these genes.

Understanding regulation of fatty acid metabolism and FAS gene expression in a *fatb* mutant of Arabidopsis

Chapter 3 takes a different approach and centers on the characterization of an Arabidopsis *FATB* knock-out mutant (*fatb-ko*). FATB belongs to the acyl-ACP thioesterase family of proteins and alteration of its function by either mutation or over-expression has proved to be instructive in disclosing mechanisms of regulation of fatty acid metabolism (Eccleston and Ohlrogge, 1998; Ohlrogge *et al.*, 1995). These observations together with our interest in understanding production of saturated fatty acids in plants and their role in development, prompted us to characterize the *fatb-ko* mutant. Thus, Chapter 3 describes the central role of FATB in producing saturated fatty acids and the essential role of these molecules in plant growth and development. In addition, analysis of saturated fatty acid derivatives provides evidence for the involvement of FATB in supplying fatty acids to distinct biosynthetic pathways and how cells control the economy of saturated fatty acids. These results provide clues to understand the effects of reduced saturated fatty acids on plant growth and set the basis for the experiments describe in Chapter 4.

Chapter 4 illustrates the importance of altering acyl-ACP thioesterase expression in disclosing regulatory signals and mechanisms for regulation of fatty acid metabolism. *fatb-ko* leaves respond to low levels of saturated fatty acids by increasing the rate of fatty acid synthesis by 40 % compared to wild type. Moreover, western blot analysis revealed that BCCP, ACPs and 18:0-ACP desaturase levels are increased by 1.5- to 2-fold in mutant leaves compared to wild type. Thus, higher rates of fatty acid synthesis are achieved at least in part by increasing fatty acid gene expression. In summary, plant cells appear to have mechanisms that sense and respond to subnormal levels of saturated fatty

acids in the cytoplasm. These mechanisms probably coordinate the requirements for lipid synthesis in the cytosol and fatty acid production inside the plastids. Moreover, the same mechanisms may also activate expression of nuclear fatty acid genes.

With the purpose of introducing the role of acyl-ACP thioesterases in fatty acid synthesis in plants together with the role of fatty acids in the synthesis of different cellular components and the current knowledge in regulation of fatty acid synthesis, a brief overview of these topics is given below.

Fatty acid synthesis in plants

Plants are fundamentally different from other eukaryotes in the molecular organization of the enzymes of fatty acids biosynthesis (FAS). The individual enzymes of this primary pathway are dissociable soluble components located in the stroma of the plastids whereas in other eukaryotes fatty acid synthesis is catalyzed by multifunctional polypeptide complexes located in the cytosol (Somerville *et al.*, 2000; Schuller *et al.*, 1992; Chirala, 1992).

The central carbon donor for fatty acid biosynthesis is the malonyl-CoA produced by acetyl-CoA carboxylase (ACCase). In dicot plants, plastidic ACCase is a mutisubunit enzyme composed of four dissociated polypeptides (biotin carboxyl carrier protein [BCCP], biotin carboxylase [BC], and α- and β-carboxyl transferases (CT). BCCP, BC and α-CT subunits are nuclear encoded whereas β-CT is plastome encoded (Sasaki *et al.*, 1993). Before entering the fatty acid biosynthetic pathway, malonyl-CoA is transferred

from coenzyme-A (CoA) to an acyl carrier protein (ACP) by malonyl-CoA:ACP transacylase. From this point on, all the reactions of the pathway involve ACP (Figure 2). After being transferred to ACP, the malonyl group enters a series of reactions that result

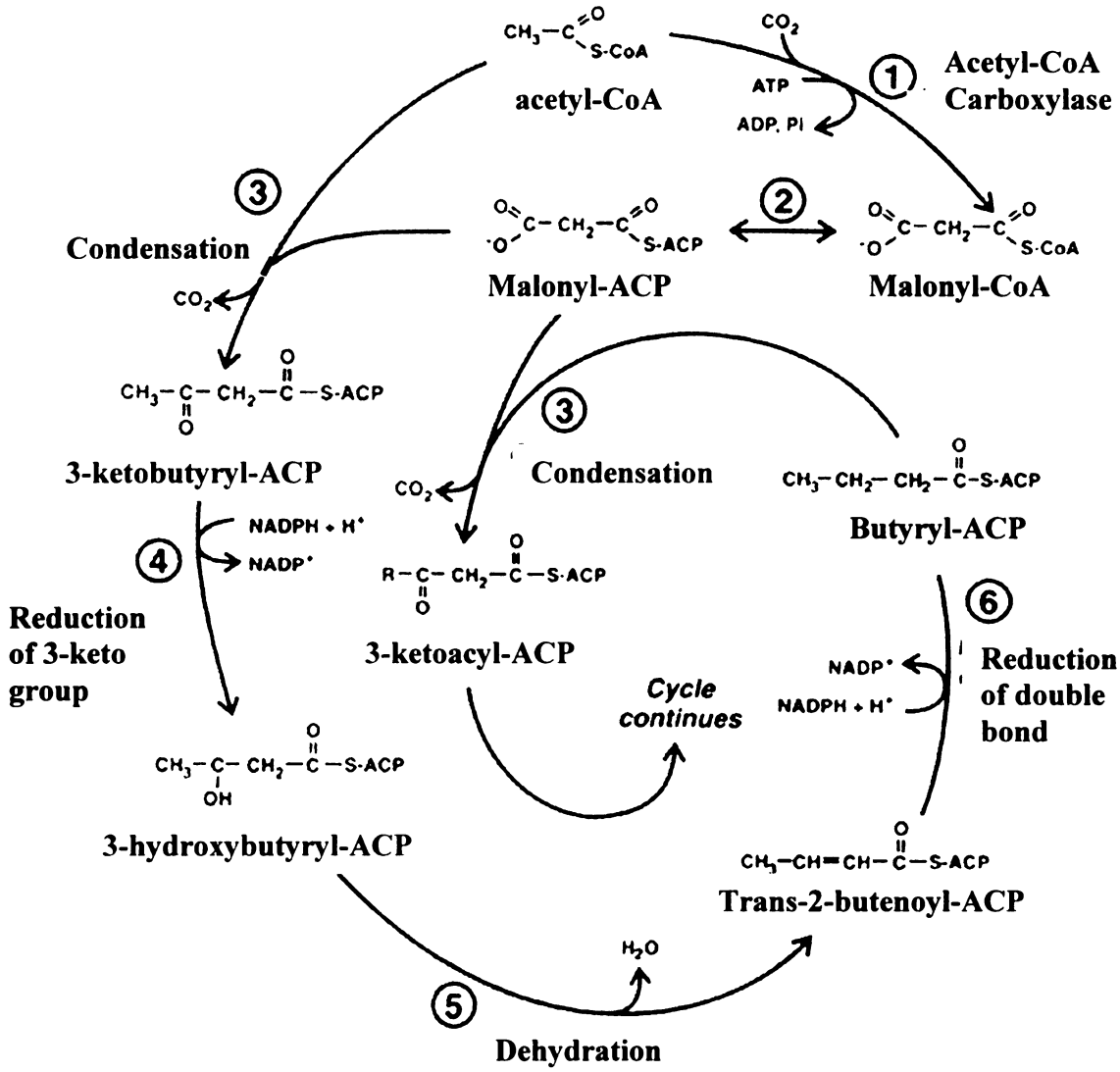


Figure 2. Simplified representation of the fatty acid biosynthesis pathway in Arabidopsis (according to Ohlrogge and Browse, 1995).

1, Acetyl-CoA carboxylase; 2, Malonyl-CoA:ACP transacylase; 3, 3-Ketoacyl-ACP synthase (KAS); 4, 3-Ketoacyl-ACP reductase; 5, 3-Hydroxyacyl-ACP dehydratase; 6, 2,3-trans-enoyl-ACP reductase.

in the formation of a carbon-carbon bond and in the release of CO_2. These reactions are catalyzed by plastidial fatty acid synthase, which in plants is also a multisubunit enzyme composed of 3-ketoacyl-ACP synthases I, II and III, 3-ketoacyl-ACP reductase, 3-ketoacyl-ACP dehydrase, and enoyl-ACP reductase (Figure 2) (Somerville *et al.*, 2000). All of fatty acid synthase subunits are nuclear encoded. The FAS pathway produces saturated fatty acids, however more than 75 % of plant fatty acids are unsaturated. The first double bond is introduced by a soluble 18:0-ACP desaturase in the plastid. Additional double bonds in fatty acids are incorporated by membrane-bound desaturases that act once the fatty acid has been incorporated into glycerol (Browse and Somerville, 1991).

The elongation of fatty acids is terminated when the acyl group is removed from ACP. This reaction can be catalyzed by acyl-ACP thioesterases that hydrolyze the acyl-ACPs to release free fatty acids and ACPs or alternatively, the acyl group can be transferred to glycerol-3-phosphate or monoacylglycerol-3-phosphate by the action of acyl-ACP acyltransferases (Figure 3) (Somerville *et al.*, 2000). More than half of the fatty acids produced in the plastids flow through acyl-ACP thioesterases and are exported from this organelle in the form of acyl-CoAs. These molecules are later used for glycerolipid synthesis at the level of the endoplasmic reticulum (ER). Based on its similarity to the animal pathway for glycerolipid synthesis it is named Eukaryotic Pathway (Figure 4). The fraction of fatty acids that remains in the plastid is used for glycerolipid synthesis within the plastid and based on its similarity to the bacterial pathway it is named the Prokaryotic Pathway (Figure 4). Some of the lipids synthesized by the Eukaryotic

Figure 3. Schematic representation of the enzymatic reaction catalyzed by acyl-ACP thioesterases in plants.

Acyl-ACP thioesterases or FAT (fatty acid thioesterases) enzymes catalyze the hydrolysis of the thioester bond between acyl carrier proteins (ACP) and fatty acids to release free ACPs and free fatty acids inside the plastids. Fatty acid are then exported from the plastid as acyl-CoA molecules.

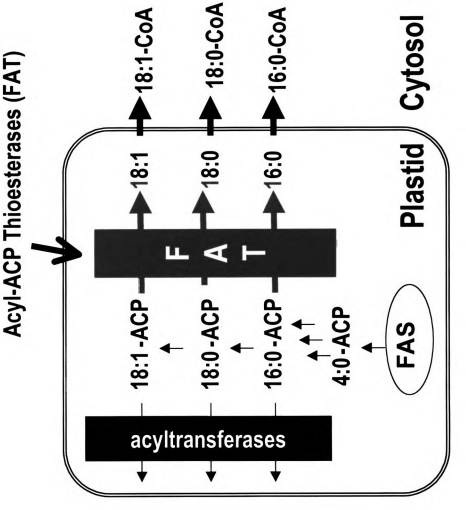
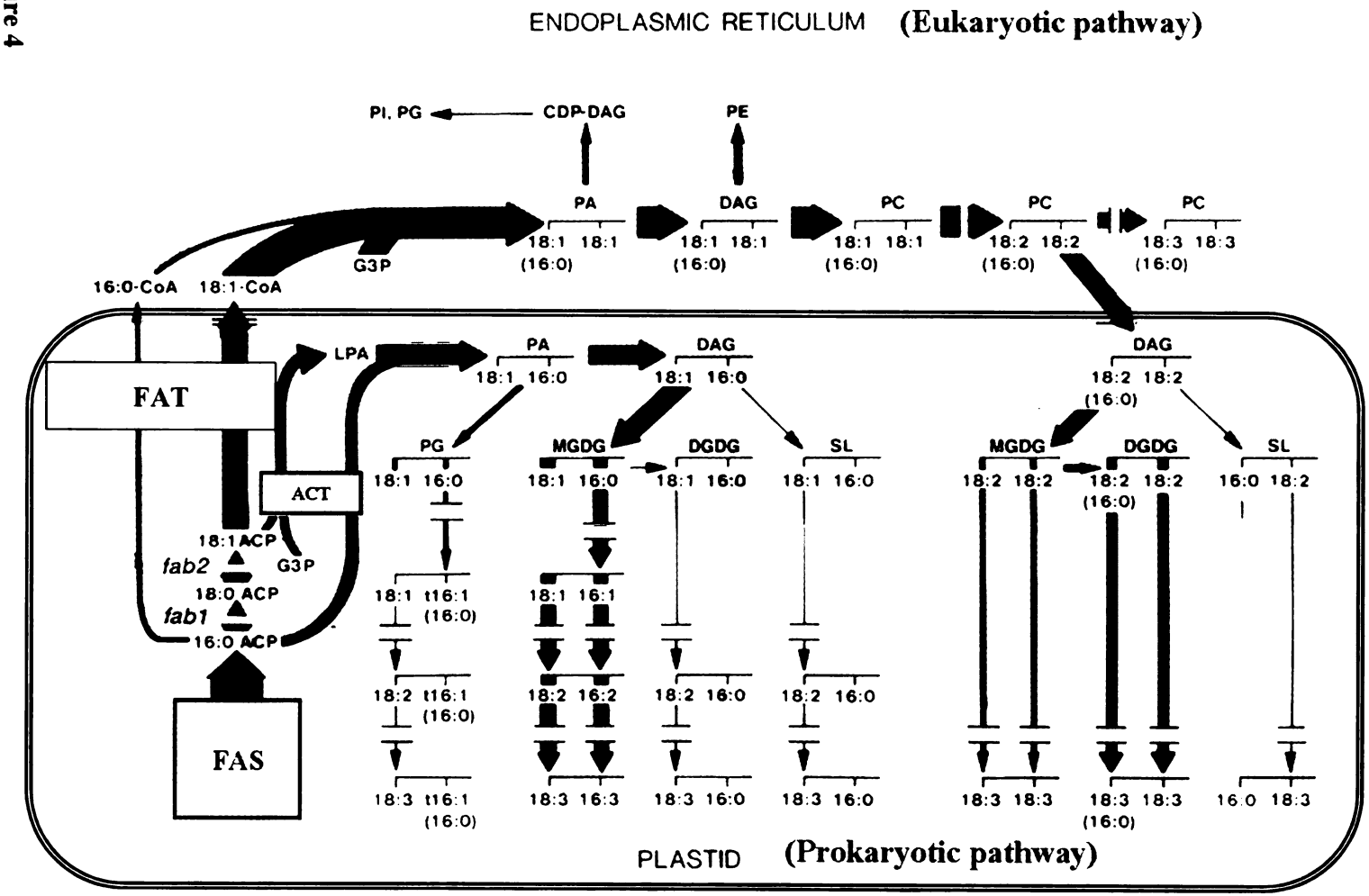

Acyl-ACP Thioesterases (FAT)
18:1-CoA
18:0-CoA
16:0-CoA
Cytosol
Plastid
18:1
18:0
16:0
F
A
T
18:1 -ACP
18:0 -ACP
16:0 -ACP
4:0 -ACP
FAS
acyltransferases

Figure 3

Figure 4. Simplified scheme of the glycerolipid biosynthetic pathway in Arabidopsis (adapted from Browse and Somerville, 1991).

Fatty acid synthesis (FAS) takes place inside the plastid and its acyl-ACP products (16:0-ACP, 18:0-ACP and 18:1-ACP) are substrates of either acyl-ACP thioesterases (FAT) or acyl-ACP acyltranferases (ACT). By the action of the latter, acyl groups enter the Prokaryotic Pathway of lipid synthesis inside the plastids to produce MGDG, DGDG, PG and SL. By the action of acyl-ACP thioesterases, acyl groups leave the plastid as acyl-CoA molecules and become substrates of the Eukaryotic pathway of lipid synthesis in the endoplasmic reticulum (ER). Fatty acids can return to the plastid in the DAG moiety of PC and are used for the synthesis of MGDG, DGDG and SL within the plastid. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SL, sulfolipid; DAG, diacylglycerol; PA, phosphatidic acid; LPA, lysophosphatidic acid.

Figure 4


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

Pathway traffic back to the plastid and intermix with the plastid lipid pool (Figure 4) (Browse and Somerville, 1991).

The role of fatty acids in the synthesis of different cellular components

Membrane glycerolipids

Membrane glycerolipids have fatty acids attached to the *sn*-1 and *sn*-2 positions of the glycerol backbone and a polar head group in the *sn*-3 position. The combination of non-polar acyl chains with polar head groups leads to the amphipathic physical properties of these molecules, which are essential for the formation of membrane bilayers (Nagle *et al.*, 2000). Fatty acids are carboxylic acids of highly reduced hydrocarbon chains that in plants can range from 8 to 32 carbons in length. However, the major fraction of membrane glycerolipids contain chains of 16 and 18 carbons. Shorter and longer fatty acids are usually found in storage lipids or epicuticular waxes (Post-Beittenmiller, 1996). Fatty acids in lipid membranes contain from one to three *cis* double bonds and five of these molecules (18:1, 18:2, 18:3, 16:0 and 16:3) make up over 90 % of membrane glycerolipid acyl chains. The number and position of double bonds in fatty acids have profound effects on their biophysical properties. For example, incorporation of a *cis* double bond between carbons 9 and 10 of an 18 carbon saturated fatty acids decreases its melting temperature from 70° C to 14° C. The addition of a second *cis* double bond between carbons 12 and 13 reduces its melting point even further, from 14° C to –5° C.

The structure of the head group that is incorporated in the *sn-3* position of glycerolipids defines seven different classes of these molecules (e.g., phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI)). In addition, each class is composed of distinct molecular species defined by the fatty acids attached to them. For example, PC carries either a 16 carbon saturated fatty acid in the *sn-1* position and an 18 carbon unsaturated in the *sn-2* or unsaturated fatty acids in both the *sn-1* and *sn-2* positions. The fatty acid composition of individual glycerolipids is critical to confer the required biophysical properties to cell membranes (Mc Elhaney, 1989). Those properties are essential for fluidity, permeability, vesiculation, transport and protein activity (e.g., photosynthesis). Moreover, crystallization of intrinsic membrane proteins revealed that specific lipids co-crystallize with them and are essential parts of their structures and functions (Katona *et al.*, 2003; Murtazina *et al.*, 2002).

One central issue with respect to the role of glycerolipids in membranes is framed by the observation that each membrane of the cell has a characteristic and distinct complement of glycerolipids and that, within a single membrane, each class of glycerolipid has a distinct fatty acid composition (Somerville *et al.*, 2000). Furthermore, the composition of each membrane and lipid class is largely conserved throughout the plant kingdom. This conservation implies that differences in lipid structure are important for membrane function. However, the details of this structure/function relation have remained elusive, in part because plants can adapt to significant changes in lipid composition and the effects of those changes are not always obvious. For example, highly unsaturated 18:3 and 16:3 fatty acids constitute approximately 70 % of thylakoid membrane fatty acids.

Surprisingly, an Arabidopsis triple mutant *fad3fad7fad8* which completely lacks 18:3 and 16:3 exhibits normal rates of vegetative growth and photosynthesis at 22° C. These results demonstrate that these polyunsaturated fatty acids are not essential for photosynthesis in plants grown at standard conditions. The loss of 18:3 and 16:3 have noticeable effects only at low (below 10°C) and high (above 30°C) temperatures (Wallis and Browse, 2002).

Other fatty acid-derived products

In addition to their role in membrane glycerolipid biosynthesis, fatty acids are used for several other biosynthetic pathways in plant cells. At the level of the ER, synthesis of sphingoid bases and ceramides consume two molecules of fatty acids. Ceramides are later glycosylated at the level of the ER and Golgi apparatus to form the essential lipids called sphingolipids. These lipids are found in the outer leaflet of the plasma membrane and have critical functions in vesiculation, Golgi trafficking and signaling (Sperling and Heinz, 2003). Similarly to glycerolipids, a large number of sphingolipid species exist that differ in the number of double bonds and hydroxyl groups in the sphingoid base, type of acyl group in the ceramide moiety and type and number of sugars in the head group (Sperling and Heinz, 2003). Most of the knowledge about sphingolipid function in plants has been inferred from information in animals and yeast and little direct experimental data exist on their role in plants.

Fatty acids are also used in epidermal cells for the synthesis of epicuticular waxes and cutin. Waxes are derived only from saturated fatty acids that are elongated at the level of the ER to chains up to 32 carbons and later modified to alkanes, aldehydes, alcohols and ketones (Post-Beittenmiller, 1996). Waxes are deposited outside cells and create an hydrophobic barrier that protects plants from water loss and environmental stresses. Cutins are derived from saturated and unsaturated fatty acids that become polyhydroxylated and crosslinked to form a mesh that confers mechanical resistance to the outer surface of plants (Kolattukudy, 2001). In seeds, fatty acids are precursors of triacylglycerol (TAG) biosynthesis, the major sink of carbon in oilseeds. In addition, fatty acids are the substrates for all sort of acylation reactions (e.g., proteins, sterols, etc) and also precursors of signaling molecules (Farmer *et al.*, 2003).

Regulation of fatty acid biosynthesis in plants

Regulation by light

The carboxylation of acetyl-CoA to produce malonyl-CoA by acetyl-CoA carboxylase (ACCase) is the first committed step of fatty acid synthesis and this enzyme is considered most responsible for the primary regulation of this pathway (Ohlrogge and Jaworski, 1997). In animals and other non-plant systems, the activity of ACCase has been determined to represent a primary regulatory step (Wakil *et al.*, 1983; Goodridge, 1972). Fatty acid synthesis is 10-fold or higher in illuminated leaves than non-illuminated ones (Bao et al., 2000; Browse *et al.*, 1981). Some of the light-dependence of this pathway can be attributed to changes in the stromal pH, Mg2+, ATP/ADP ratio and reductants (Hunter

and Ohlrogge, 1998; Sasaki *et al.*, 1997; Eastwell and Stumpf, 1983). The in vitro activity of ACCase is sensitive to all these conditions and in addition the formation of malonyl-CoA is light dependent. Thus, evidence suggests that light affects ACCase activity and subsequently the rate of fatty acid production (Post-Beitenmiller *et al.*, 1991). However, the mechanism by which ACCase is activated by light is still under debate. The β-CT subunit of ACCase is phosphorylated at a serine residue in the light and evidence points to the β-CT of chloroplast ACCase as a candidate for regulation by protein phosphorylation/dephosphorylation (Savage and Ohlrogge, 1999).

Regulation by feedback inhibition

Most biochemical pathways are controlled in part by feedback mechanisms in which inhibition of enzyme activity occurs when the product of a pathway builds up to levels in excess of need. In most cases this inhibition occurs at a regulatory enzyme which is often the first committed step of the pathway.

In animals and yeast, it has been considered that fatty acid synthesis is partially controlled by feedback on ACCase by long chain acyl-CoAs (Wakil *et al.*, 1983). However, this model has been questioned because acyl-CoA binding proteins are in concentration high enough to sequester most acyl-CoA molecules and therefore would be present only at extremely low levels (Knudsen *et al.*, 1994; Rasmussen *et al.*, 1993). Shintani and Ohlrogge (1995) demonstrated that fatty acid synthesis is inhibited by feedback mechanisms in plant cells. However, potential feedback inhibitors such as acyl-

CoA, free fatty acids and glycerolipids failed to strongly inhibit plastidial ACCase at physiological concentrations *in vitro*. Because fatty acid synthesis takes place inside the plastid but the major utilization of its products is at the ER membranes, it is likely that feedback regulation must allow communication across the plastid envelope. At this time it is not known what molecules are involved in feedback regulation of plastidial fatty acid synthesis.

Regulation by demand

Evidence that utilization of fatty acids can increase rates of fatty acid biosynthesis comes from different experiments. First, when a plant 12:0-ACP thioesterase (MCTE) is over-expressed in *E.coli* cells, fatty acid synthesis is increased and cultures accumulate at least 10-fold more total fatty acids than control cultures. In these experiments, the removal of the products of fatty acid synthesis (acyl-ACPs) to free fatty acids by MCTE, appeared to release feedback inhibition on ACCase, resulting in higher malonyl-CoA and fatty acid production (Ohlrogge *et al.*, 1995). Second, over-expression of cloned membrane proteins results in an increased production of fatty acids for membranes to accommodate the excess proteins and maintain lipid/protein ratios constant (Nieboer *et al.*, 1996). Therefore, the rate of fatty acid synthesis can apparently be regulated by the demand for fatty acids needed for membrane synthesis. Finally, analysis of developing *Brassica napus* seeds expressing high levels of the California Bay 12:0-ACP thioesterase (MCTE) shows that expression of fatty acid synthesis and β-oxidation enzymes were induced. These seeds appear to produce more 12:0 that can be metabolized to TAG and the excess

12:0 is degraded. To compensate for the loss of carbon by β-oxidation, cells increase fatty acid production. Moreover, protein and activity levels of ACP, ACCase, 18:0-ACP desaturase, and KAS III increase 2 to 3-fold, indicating that concerted induction of these enzymes has occurred (Eccleston and Ohlrogge, 1998).

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CHAPTER 2

DIFFERENTIAL REGULATION OF MESSENGER RNA LEVELS OF ACP ISOFORMS IN ARABIDOPSIS

ABSTRACT

All higher plants express several different acyl carrier protein (ACP) isoforms in a tissue specific manner. We provide evidence that expression of mRNA for the most abundant ACP isoform in Arabidopsis leaves (ACP4) is increased several fold by light whereas mRNA levels for ACP isoforms 2 and 3 are independent of light. The presence of GATA-like motifs in the upstream region of the *Acl1.4* gene (encoding ACP4) and the similarity in light-mediated induction to ferredoxin-A mRNA suggests a direct role of light in *Acl1.4* gene activation. Polyribosomal analysis indicated that light also affects the association of ACP transcripts with polysomes, similarly to mRNAs encoding ferredoxin-A. ACP2, ACP3 and ACP4 mRNA levels were also examined in Arabidopsis cell-suspension culture and found to be differentially controlled by metabolic and/or growth derived signals. Moreover, similar mechanisms operate *in planta* for *ACP2* gene expression. Fusion of the ACP2 promoter to luciferase demonstrated that regulation of ACP2 mRNA levels was transcriptional and responded to growth/cell-cycle signals. Arabidopsis ACP2 promoter deletion analysis in tobacco cells revealed conserved mechanisms of ACP gene expression between plant species and identified promoter regions important for transcriptional regulation. Comparison of Arabidopsis ACP promoters disclosed the presence of 2 conserved elements, an 11 bp element found in the five Arabidopsis *ACP* genes and a 6 bp element found only in ACP2 and 3. Comparison of proximal ACP 5' upstream sequences between diverse plant species showed that two

motifs have been conserved during evolution, a CTCCGCC box and C-T rich sequences. In Arabidopsis these regions are transcribed and are present in the 5' untranslated regions (UTRs) of ACP mRNAs. We tested the role of ACP1 and ACP2 mRNA 5'-leader regions in transgenic Arabidopsis plants and demonstrated that they enhanced several-fold the expression of a reporter gene in a tissue specific manner. By using large scale mRNA profiling techniques we demonstrated that fatty acid and lipid synthesis genes did not respond to inhibition of fatty acid synthesis in Arabidopsis cells.

INTRODUCTION

Despite our current understanding of the biochemistry of fatty acid and lipid synthesis in plants, the signals and factors that direct the expression of genes in these pathways remain largely unknown. Unlike in other organisms, the enzymes responsible for *de novo* fatty acid synthesis in plants are not localized in the cytosol but rather in the plastid. Although a portion of the newly synthesized acyl chains is utilized for lipid synthesis within the plastid, a majority is exported into the cytosol for glycerolipid assembly at the endoplasmic reticulum (ER) (Somerville *et al.*, 2000). In addition, some extraplastidial glycerolipids return to the plastid and extensive lipid interchange exists between these two organelles (Ohlrogge and Jaworski, 1997). In order to adjust to changes in the demand of lipids during tissue development and environmental influences, plant cells must regulate and coordinate the expression of the many genes involved in fatty acid and lipid synthesis pathways. An attractive possibility is the existence of a global transcriptional control for these genes, perhaps comparable to the yeast system (Schuller

et al., 1992). For example, it has been shown that the expression of the different subunits of acetyl-CoA carboxylase (ACCase) is orchestrated during tissue development (Ke *et al.*, 2000). This enzyme catalyzes the first committed step in fatty acid synthesis and available evidence points to its key role as a major regulatory enzyme in fatty acid production (Ohlrogge and Jaworski, 1997). In addition, global post-transcriptional mechanisms have also been suggested for regulation of fatty acid synthesis genes in plants (Eccleston *et al.*, 1998).

Analyses of gene expression under conditions where the demand for fatty acids is elevated may unravel part of the signals and mechanisms that participate in the regulation of genes involved in plant lipid biosynthesis. A situation where the cells are fully engaged in fatty acid production is during leaf expansion in illuminated plants (Browse *et al.*, 1981). In addition to cell division and growth, light induces the differentiation of the proplastid to the specialized, membrane-rich chloroplast (Kasemir, 1979). This transition involves not only a radical change in the structure and function of the plastid but also its increase in size and number per cell. Therefore, *de novo* synthesis of glycerolipids is critical to provide chloroplasts with new lamellae to accommodate the photosynthetic apparatus. During the process of leaf expansion light activates transcription and translation of a large number of genes that participate in chloroplast differentiation (Chory *et al.*, 1994). Moreover, the interdependent relation between chloroplast development and the activation of nuclear genes has been confirmed by mutant analyses in Arabidopsis (Susek *et al.*, 1993). As a consequence of the plastidic location of the fatty acid synthesis machinery and its commitment to supplying lipids for new membrane

synthesis, it seems possible that at least some of the genes participating in this pathway belong to the group of genes activated by light during leaf expansion. However, previous studies argue against this hypothesis and suggest a minor role of light on the regulation of the genes for fatty acid synthesis (Scherer *et al.*, 1987; Battey *et al.*, 1990; Baerson *et al.*, 1993). Presently, the only gene known to participate in glycerolipid production and to be transcriptionally induced by light in Arabidopsis is the chloroplast ω-3 fatty acid desaturase (FAD7) (Nishiuchi *et al.*, 1995).

A second condition where the fatty acid synthesis machinery is very active is during the exponential growth of plant cells in culture. When plant cells are grown in liquid culture in the presence of a carbon source, they show rapid incorporation of labeled precursors into phospholipids and neutral lipids (Weber *et al.*, 1992). The presence of an energy source such as carbohydrates in the media increases the cell growth rate and hence the need for new membranes. Sugars not only function as substrates for growth but also affect sugar-sensing systems that initiate changes in gene expression. In plant metabolism, carbohydrate depletion generally enhances the expression of genes involved in photosynthesis, reserve breakdown and export, whereas abundant carbon resources favor genes for storage and utilization (Koch, 1996). For example, Graham *et al* (1994) showed that in cucumber, the mRNA levels for two enzymes of the glyoxylate cycle (malate-synthase and isocitrate-lyase) are coordinately induced not only during post-germinative seedling development but also by a metabolic signal derived from sugars. The glyoxylate cycle plays an important role in the degradation of fatty acids during seedling germination. In animal pancreatic cells a glucose-mediated signal activates the

transcription of the acetyl-CoA carboxylase gene (Brunt *et al.*, 1993). Thus, due to the metabolic regulation via sugars of several genes involved in primary biochemical pathways in plants, it is likely that at least some of the genes involved in fatty acid synthesis are also under the control of similar mechanisms.

Finally, in bacteria inhibition of fatty acid biosynthesis by drugs triggers the transcriptional activation of genes involved in fatty acid and lipid metabolism (Schujman *et al.*, 2003). Thus, bacteria have the ability to sense a decrease in the activity of the FAS pathway and to respond by adjusting the expression of FAS genes.

The regulation of acyl carrier proteins (ACPs) has been the focus of several studies based on their central role in lipid biosynthesis in plants (Hannapel *et al.*, 1988; Hlousek-Radojcic *et al.*, 1992, Baerson *et al*, 1993). ACPs are small acidic proteins that carry the nascent acyl chains during the synthesis of 16 and 18 carbon acyl groups. In this report we demonstrate that the transcripts for several isoforms of Arabidopsis ACPs are subject to differential regulation by light and also by metabolic and/or growth control. In addition, we provide evidence for the role of the ACP1 and ACP2 5' untranslated sequences (UTRs) in the enhancement and tissue specific expression of a reporter gene in Arabidopsis plants.

RESULTS

ACP4 mRNA Levels are Increased by Light

The participation of light in the activation of fatty acid synthesis in young leaves is well established (e.g. Browse *et al.*, 1981; Bao *et al.*, 2000). Light regulation can be attributed in part to activation of ACCase by mechanisms that involve reduced thioredoxin, phosphorylation, substrate activation, cofactors and pH changes (Sasaki *et al.*, 1997; Hunter and Ohlrogge, 1998; Savage *et al.*, 1999). Although light-mediated mechanisms of gene activation such as transcription are prominent during chloroplast biogenesis in leaves, no available evidence connects these mechanisms to the activation of genes responsible for *de novo* fatty acid synthesis. Therefore, we first examined the influence of light on the mRNA steady state levels of distinct isoforms of Arabidopsis acyl carrier proteins.

Based on protein analyses, ACP1, ACP2 and ACP3 have been identified in all tissues examined (Hlousek-Radojcic *et al.*, 1992) whereas a fourth isoform (ACP4) is expressed predominantly in leaves (Shintani, 1996). As shown in Figure 5, the mRNA levels of ACP4 increased approximately 4-fold in leaf tissue when two week-old *Arabidopsis* seedlings were reilluminated for 4 hours after a 24 hours dark period. An even higher increase (5-fold) was observed at eight hours of illumination with a decline to 4-fold again after 12 hours (Figure 5). Interestingly, the ACP4 messenger RNA presented similar kinetics of light-mediated induction to the Arabidopsis ferredoxin-A (FEDA) mRNA, a 4-fold increase after 4 hours in white light with a subsequent decline after 12 hours (Figure 5). Previous studies demonstrated that the gene for FEDA is transcriptionally activated by light, and part of this induction is initiated by phytochromes

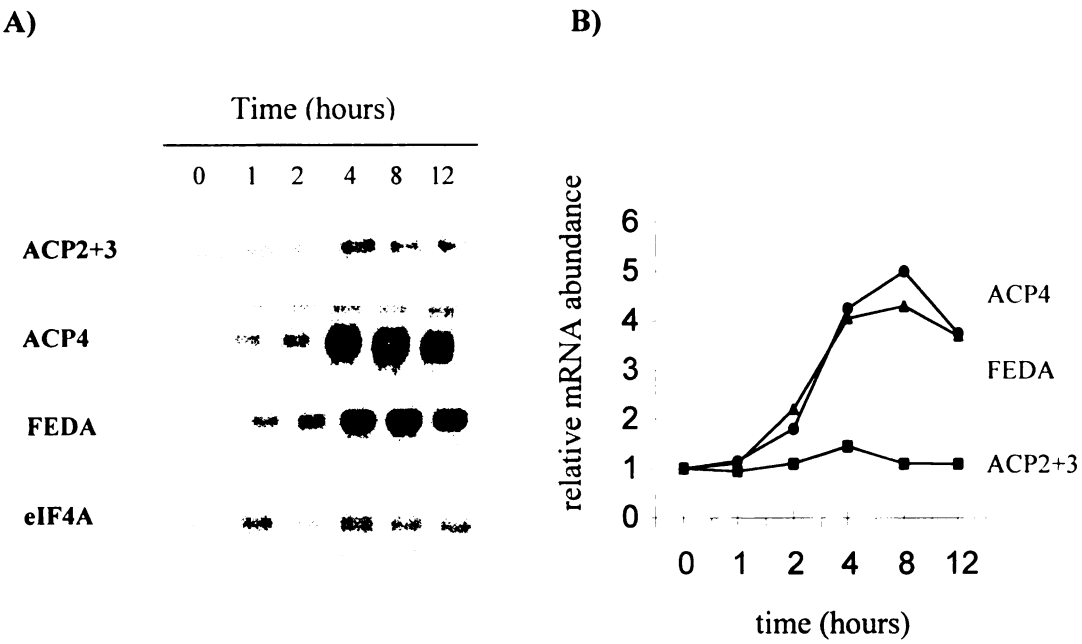

Figure 5. Light-mediated induction of ACP4 mRNA levels in Arabidopsis leaf tissue.
(A) Northern blot analyses of two week-old Arabidopsis plants dark treated for 24 hours and reilluminated with fluorescent white light for the times indicated. Each lane of the blot contains 5 μg of total RNA from leaf tissue. The eIF4A probe was used as a loading control. (B) Densitometry scanning of the blots in A. The signal intensities at each time were normalized with the corresponding eIF4A signal and expressed relative to time 0 (set arbitrarily to one).

(Caspar *et al.*, 1993).

In contrast to ACP4 transcript levels, the signal presented by a probe corresponding to the ACP2 coding sequence did not show intensity variations in the conditions tested (Figure 5). This result agrees with a previous study that showed that the Arabidopsis *Acl1.2* gene promoter (ACP2) does not confer light responsiveness to a reporter gene in transgenic

tobacco seedlings (Baerson *et al.*, 1993). Because the ACP2 and ACP3 isoforms are 82% similar at the nucleotide level, we assumed that the signal conferred by the ACP2 fragment represented the abundance of both, ACP2 and ACP3 transcripts (ACP2+3 in Figure 5). This assumption is also based on the observation that both proteins are expressed in similar amounts in Arabidopsis leaf tissue (Hlousek-Radojcic *et al.*, 1992) and that the corresponding promoters confer similar levels of expression to a reporter gene in Arabidopsis leaves (Baerson *et al.*, 1998).

Polyribosomal Association of ACP mRNAs is Increased by Light

Previous studies suggested that the expression of ACPs could be regulated at the level of translation. First, transgenic *Brassica napus* plants over-expressing a 12:0-ACP thioesterase show an increase of approximately 2-fold in the ACP protein level with no significant changes in the corresponding messenger abundance (Eccleston *et al.*, 1998). Second, Hannapel *et al* (1988) reported that during seed development in soybean, the relative abundance of ACP and lectin mRNAs is at most 28-fold different whereas the corresponding protein levels differ at least 200-fold, suggesting a differential translational efficiency between these two mRNAs.

Based on these observations, we compared the polyribosomal distribution of ACP mRNAs with other transcripts highly expressed in leaf tissue of Arabidopsis plants. In addition, the fact that light has a major effect on the polyribosomal association of several messengers RNA encoding plastidic proteins (Berry *et al.*, 1990; Dickey *et al.*, 1998)

prompted us to investigate the influence of light on the association of ACP mRNAs with ribosomes. Therefore, we analyzed Arabidopsis leaf tissue from plants either dark treated for 24 hours or reilluminated for 12 hours with

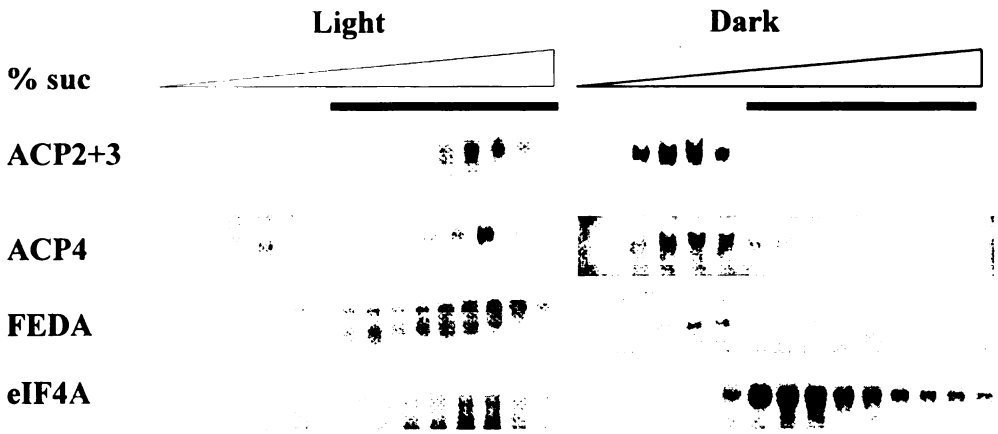

Figure 6. Light affects the polyribosome association of ACP mRNAs.
Leaf tissue extracts from plants grown in the dark for 24 h (Dark) or reilluminated for 12 h after a 12 h dark period (Light) were spun in 15 to 60 % sucrose gradients. Sixty aliquots were collected at identical volume intervals and absorbance at 260 nm measured. Total RNA was extracted from 15 fractions of the gradients, loaded onto an agarose gel and analyzed by Northern blot. The blots were hybridized with probes corresponding to the sequences indicated. Fractions 7 to 15 correspond to the polyribosomal fractions (black line).

white fluorescent light. The results demonstrated that the messengers for ACP2+3 and ACP4 were associated with polyribosomes (fractions 10 to 15 of the gradient) after 12 hours of illumination (Figure 6). In contrast, after 24 hours in the dark, the transcripts for the ACP isoforms appeared in the upper fractions (3 to 6) of the gradient, corresponding to low molecular weight polyribosomes or ribosome-free mRNAs (Figure 6). The polyribosomal distribution of the ferredoxin A (FEDA) mRNA was similar to that of ACP mRNAs in both conditions (Figure 6). The eukaryotic initiation factor 4A (eIF4A)

transcript also showed association with polyribosomes in the light, but in contrast to ACP and FEDA mRNAs, a significant proportion of the eIF4A transcript was still bound to polyribosomes after 24 hours in the dark (Figure 6). In summary, these observations indicate that the transcripts encoding for ACP isoforms are associated with polyribosomes in Arabidopsis leaf tissue in a light-dependent manner similar to FEDA but differ significantly with respect to eIF4A mRNAs in the dark.

Polyribosomal association of ACP mRNAs in developing seeds of wild type *B.napus* and transgenics over-expressing MCTE

To evaluate if translation of ACP mRNAs was altered in developing seeds of transgenic *Brassica napus* over-expressing a medium-chain thioesterase (MCTE) compared to wild type, the polyribosomal distribution of ACP mRNAs was analyzed in seeds of both plant classes. Flowers from wild type plants and transgenic *B. napus* (event 198, Voelker *et al.*, 1992) were tagged and developing seeds harvested at 21, 28 and 35 days after flowering. These time points correspond to mid-stage development of *B.napus* seeds, the period of active oil biosynthesis and accumulation.

The distribution profile of ACP transcripts on polysomes was similar between *B. napus* wild type and transgenic seeds during the three developmental stages analyzed (Figure 7). These results suggested that translation of ACP mRNAs in seeds was not affected by over-expression of MCTE and mechanisms affecting protein stability were more likely explanations for higher FAS protein levels in transgenic seeds (Eccleston and Ohlrogge,

1998). In addition, association of ACP transcripts with high molecular weight polysomes indicated that ACP mRNAs were efficiently translated in seeds.

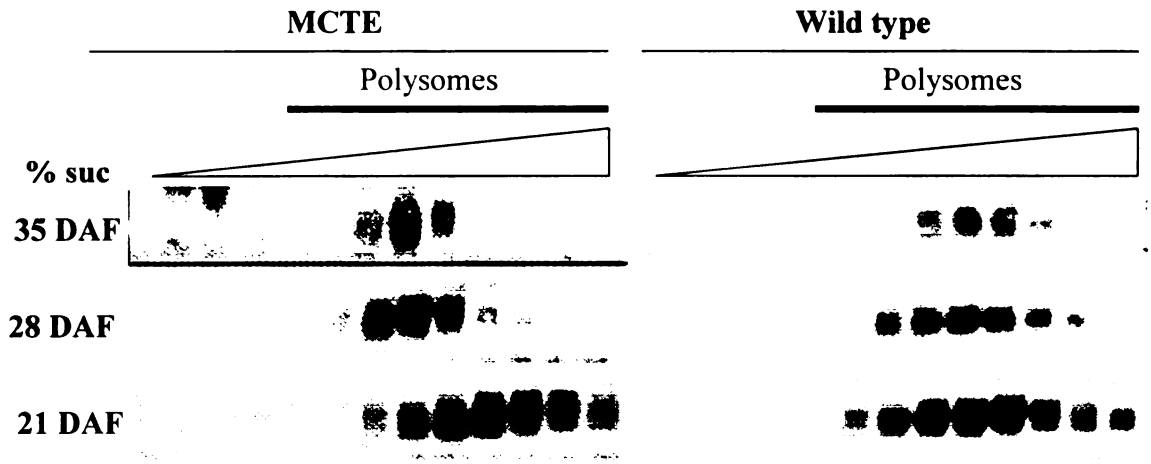

Figure 7. Polysome distribution of ACP mRNAs in wild type and transgenic (MCTE) *B. napus* developing seeds.

Developing seeds from wild type *B.napus* and transgenic overexpressing MCTE were harvested at 21, 28 and 31 days after flowering (DAF). Whole seeds were lised and extracts loaded onto 15-60 % sucrose gradients. Fractions containing polysomes were collected from the gradients after centrifugation and total RNA extracted and resolved by electrophoresis. After transfer, the blots were probed with a seed specific ACP cDNA (Bn-ACP 28f10).

Conserved Motifs occur in the 5' Leader Region of ACP mRNAs

The examination of Arabidopsis genomic and cDNA ACP sequences has revealed two unusual motifs in the 5' leader region of ACP mRNAs (Table 2 and Ohlrogge *et al.*, 1991). One feature is the presence of short sequences rich in pyrimidines. The high cytosine and thymidine content is uncommon, and leader sequences of plant mRNAs tend

to be rich in adenines and thymidines (Joshi, 1987). A second conserved motif is an element composed of seven nucleotides CTCCGCC (Table 2). In addition to Arabidopsis sequences, these features are also conserved in the 5' leader region of ACP messengers from several diverse species (Table 2). The importance of 5'untranslated regions (UTRs) as essential regulators of gene expression in plants has been described (Dickey *et al.*, 1998; Bolle *et al.*, 1996).

To analyze the participation of the 5' leader sequences of the ACP mRNAs in the regulation of ACP expression, we generated independent Arabidopsis transgenic lines in which the 5' UTR sequences of the Arabidopsis ACP1 (lines atL-acp1) and ACP2 (lines atL-acp2) mRNAs were fused to the luciferase reporter gene (LUC) under the control of the CaMV35S promoter (Figure 8). To preserve the endogenous translation initiation site a short portion of the corresponding ACP coding sequence was also included (see Figure 8 for details). The 5' leader regions of ACP1 and ACP2 mRNAs present sequence variations but both have conserved CU rich regions and include the heptanucleotide motif CTCCGCC (Table 2). However, ACP1 and ACP2 transcripts contain distinct translation initiation sites (Figure 8). The AUG context in the *Acl1.1* gene (ACP1) matches with one of the most common translation initiation sequences found in plants, AAACAAUGGC whereas the translation initiation site in the *Acl1.2* gene (ACP2), CUUCUAUGGC is found in a smaller number of plant genes (Joshi *et al.*, 1997). This suggests that the efficiency of AUG recognition by the translation machinery might also influence ACP expression. A third line of transgenic plants carrying the LUC gene fused to a deleted version of the ACP1 leader region (line atL-del1) was also generated. In this case, most

Table 2. Proximal upstream sequences of ACP genes in different plant species.

A.thaliana ACP1: ctctttgtaca**CTCCGCC**ctctctcccc atctctttcg acagatctcttctctctctcgtgtttcacgaaaca atg

A.thaliana ACP2 : ctctttgtctt**CTCCGCC**tcctccgatctcactccgatctctctacgattcattcttct atg

A.thaliana ACP3: actgtttctcatctcttcgtctt**CTCCGCC**tccttcaatctcactccgatctctctacgattcattcgttct atg

A.thaliana ACP4 : ccgaagataggcctgaatctccgagaacaaac**CTCCGCC**acaaaaacagaagacttgttgcttgcgtatcataat cgacgccgtatctctacttgctcgaacaaacccaaaaagatacatatcaagagattaaaccttatccaactaagagaagccatttttatttttttgggtctctgag ttgtgtattgagcttcatctccttcaa atg

A.thaliana ACP5: acaaaatagtaattcacgctccttgaaca**ATCCGCC**atctctctcgatcagatcgata atg

B.campestris ACP-sf2: atcacgctctttgtaca**CTCCGCC**atctctctctctctcgagcagatctctctcgggaatatcgaca atg

B.campestris ACP-sf1: caagctaccatgggacatcacgctctttgtaca**CTCCGCC**atctctctccatctctctcgtgaataacgaaa atg

B.napus ACP: atcacgctctttgtaca**CTCCGCC**atctctctctccttcgagcacagatctctctcgtgaatatcgaca atg

B.napus ACP-28f10: acgctctgtaca**CTCCGCC**atctctctccattctctctcgtgagtaacgaca atg

B.rapa ACP: gacatcacgctctttgtaca**CTCCGCC**atctctctctctctcgagcagatctctctcgggaatatcgaca atg

C.glauca ACP: gcggccgctgttct**CTCCGTC**tcatttctttgtttccctctctcaaatcccagatctctctctctcgctttgtatct atg

C.sativum ACP: tatcgtcacactctttgtgct**CTCCGCC**ctcttcttgatcaataaacttttctcagatctaaactctatctatcaa atg

C.lanceolata ACP1-3: c**CTCCGTC**gtcccattttcccagctaccaa atg

C.lanceolata ACP1-1: cacgcct**CTCTCGCC**tcatttgctcgctccctccctccctccccatca atg

Spinach ACPII: atctctctcctctttctct**CTCCGCC**acattcattctcactcactttctctcccctctctctccgcttcttca atg

Spinach ACPI : aacttaatattcttactcaggataagcttctcactctctctctctctctctcttactacc atg

Barley ACP2: ccgccgc**CTCCACC**gccgc**CTCCACC**gcccaccgcgccggcctctccccctgtcccgtctccccc atg

Barley ACP1: acctaccagccggcctcttcccaccgcccccaaattctaccgagcag**CTCAGCC**ggccaaccc atg

A sequence of arbitrary length upstream the of translation initiation site was selected. The sequence (CTCCGCC) and its derivatives are indicated in uppercase. The C-T rich sequences are underlined. The ATG start codon is separated by one space at the right end of the sequences. *A.* = *Arabidopsis, B.* = *Brassica, C.* = *Coriandrum*

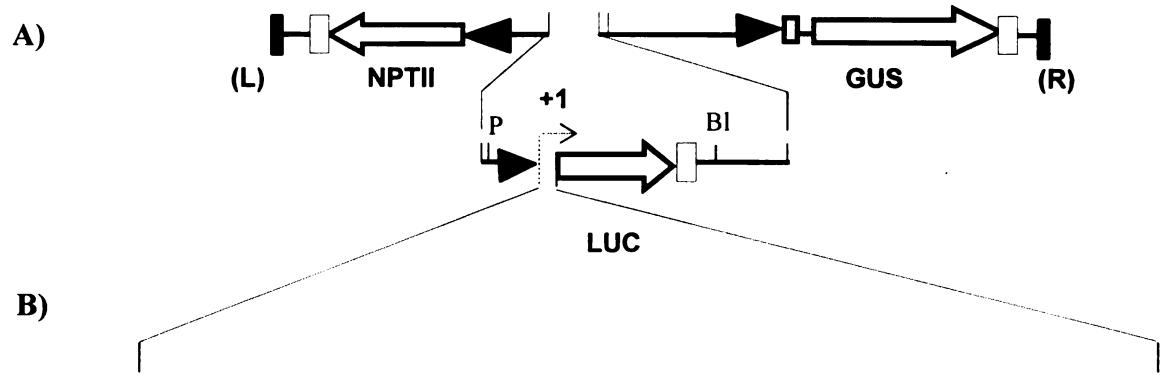

Figure 8. Constructs used for Arabidopsis transformation.
(A) Scheme of the T-DNA constructs carrying the LUC and GUS genes. Black box: T-DNA borders (L) left and (R) right ends, Open box: nopaline synthase 3' polyadenylation signal, NPTII: neomycin phosphotransferase coding sequence, black triangle: CaMV35S promoter, LUC: firefly luciferase coding sequence, GUS: β-glucuronidase coding sequence, B: BamHI, P: PstI, Bl: BglII, +1 denotes the transcription initiation site. (B) Sequences of the 5' UTRs derived from Arabidopsis ACP1 (pCaACP1), ACP2 (pCaACP2) and the deleted version of ACP1 (pCadel1) used in the three independent constructs. The ACP translation initiation codon is underlined. The displayed sequences start at the predicted transcription initiation site and therefore the leaders generated by these constructs have 8 additional bases from the vector sequence (underlined).

of the 5' UTR was removed, conserving only the first nucleotides towards the 5' end and the ACP1 translation initiation site (see Figure 8 for sequence details). As an internal control for position effect and copy number, all the T-DNA constructs also carried a cassette expressing the β-glucuronidase (GUS) gene under the CaMV35S promoter (Figure 8).

The 5' Leader Sequences of ACP1 and ACP2 Increase Reporter Gene Expression

We analyzed the LUC and GUS specific activities in expanding leaves of two week-old transgenic plants. The atL-acp1 and atL-acp2 transgenic lines showed 10- and 20-fold higher LUC/GUS ratios respectively compared to the atL-del1 lines after 24 hours in the dark (Figure 9A). From these results we conclude that the presence of the ACP 5' UTRs is essential for high expression of ACP1 and ACP2 in Arabidopsis leaves.

Based on the polyribosomal distribution of the ACP transcripts (Figure 6) we also asked whether the ACP 5' UTRs could have a light regulatory role similar to other UTRs from plastid proteins (Dickey *et al.*, 1998; Bolle *et al.*, 1994). After 6 hours of re-illumination, the activity ratio of the reporter genes in the atL-acp1 and atL-acp2 lines differed approximately 20- and 30-fold with respect to atL-del1 lines (Figure 9A). The additional increase upon reillumination in the LUC/GUS ratios suggests that light-enhanced expression of the ACP-LUC constructs is at least partly mediated by the ACP 5'UTR.

Arabidopsis developing seeds are green and posses photosynthetic capacity. However, they are considered heterotrophic as imported sucrose is their major carbon source. As shown in Figure 9B, the LUC/GUS ratios of specific activities in mid-stage developing seeds differed from the ratios observed in leaves. The activity ratio in atL-acp1 transgenic lines was approximately 2.5-fold higher than the ratio in atL-acp2 plants and 13-fold higher than the atL-del1 transgenic lines (Figure 9B). Thus, in contrast to leaf tissue, the 5' UTR of ACP1 mRNA appeared to confer a preferential expression of the LUC gene in

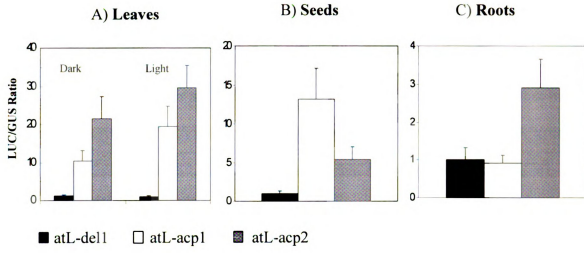

Figure 9. Reporter gene expression in different tissues of Arabidopsis transgenic plants.

atL-del1, atL-acp1 and atL-acp2 indicate the transgenic lines transformed with pCadel1, pCaACP1 and pCaACP2 respectively (see Figure 8). (A) Transgenic plants were dark treated for 24 h (Dark) or reilluminated for 6h (Light) with white fluorescent light. LUC and GUS specific activities were measured in leaves of 10 independent transgenic lines for atL-del1, atL-acp1 and atL-acp2. LUC/GUS ratios are the average of the 10 individual ratios from each transgenic line. The atL-acp1 and atL-acp2 LUC/GUS ratios are expressed with respect to atL-del1 LUC/GUS ratio (set arbitrary to one). The bars denote the standard deviation of the average. (B) LUC and GUS specific activities were measured in roots of 10 independent lines for atL-del1, atL-acp1 and atL-acp2. LUC/GUS ratios were calculated and represented as in A. (C) Reporter gene activity was measured in pools of developing seeds from 10 independent lines for atL-del1, atL-acp1 and atL-acp2. The LUC/GUS ratios were calculated and represented as in A.

developing seeds compared to the 5'UTR of ACP2 mRNA. It is noteworthy that the level of reporter gene expression in atL-acp1 lines relative to control lines was similar in leaves and seeds (between 10- and 20-fold) (Figure 9A and B).

The expression of the reporter genes was also evaluated in root tissue from 2 week-old transgenic plants. In contrast to leaf and seed tissue, the influence of the ACP1 and ACP2 5'UTRs was less pronounced and for atL-acp1 plants the ratio of reporter gene activity was similar to the ratio in atL-del1 plants (Figure 9C). This result suggests that the 5' leader of the ACP1 mRNA does not affect the expression of the LUC mRNA in roots. For atL-acp2 plants, the LUC/GUS ratio was 4-fold higher than the ratio in atL-del1 plants (Figure 9C). Similar to leaf tissue, the influence on LUC expression of the ACP2 leader was higher than the effect produced by the ACP1 leader on the same reporter gene. However, the relative ratios of reporter gene activity conferred by the ACP2 leader was between 7 and 10-fold lower in roots compared to leaves (Figure 9A and C).

ACP mRNA Levels are Affected by a Sucrose-derived Signal and/or Growth Control in Cell Suspension Cultures

In bacteria and yeast the expression of several genes involved in fatty acid and lipid synthesis is tightly coupled to growth (Jiang *et al.*, 1994; Carman *et al.*, 1999). Cells growing in the presence of a carbon source such as sucrose show high rates of transcription of these genes (Carman *et al.*, 1999). In addition to their role as energy sources, sugars have been demonstrated to control the expression of plant genes involved in diverse processes such as starch metabolism (Nakamura *et al*, 1991), storage protein accumulation (Hattori *et al*, 1990) and lipid degradation (Graham *et al*, 1994). Based on these observations, we asked whether the expression of some of the fatty acid synthesis genes in plants might also be under metabolic and/or growth control by sugars. For this purpose we analyzed the mRNA levels of ACP2, ACP3, ACP4 and the endoplasmic

reticulum (ER) associated delta-12-desaturase (FAD2) in Arabidopsis mesophyll-derived cell suspension cultures. In order to distinguish between the messengers corresponding to ACP2 and ACP3 in this experiment, we synthesized a messenger specific probe based on the 3' UTR sequences of these genes. The transcript levels of ACP2 and ACP3 isoforms declined approximately 2-fold after 48 hours of starvation (Figure 10). In contrast, the ACP4 and FAD2 transcript levels showed no significant variation during this 48 hours period (Figure 10). Thus, the absence of sucrose in the media and/or the reduced growth rate preferentially affected the expression of the ACP2 and ACP3 mRNAs. In the same experiment the transcript levels of the ribulose-1,5- biphosphate-carboxylase 1A small subunit (RBCS1A) increased approximately 2-fold after 12 hours of starvation and slightly declined afterwards (Figure 10). This result was in agreement with the fact that the gene for the small subunit of Rubisco is activated by light and repressed by sugars (Terzaghi *et al.*, 1995). To evaluate the effect of addition of sucrose to starved cells in the absence of light, the cells were grown in the dark and in the presence of 58 mM sucrose. The transcript levels of ACP2 and ACP3 increased approximately 2.5-fold after 24 hours (Figure 11). In contrast, if the cells were kept in an osmotic control media in the dark the level of the same messenger RNA remained steady (Figure 11). Interestingly, we observed that the incubation of starved cells in the dark and 58 mM sucrose had a negative effect on the ACP4 mRNA levels, decreasing its abundance more than 2.5-fold. Conversely, no variations in the levels of the same mRNA were observed with the osmotic control media in the dark (Figure 11). Thus, both the absence of light and the presence of 58 mM sucrose were responsible for the down-regulation of the ACP4

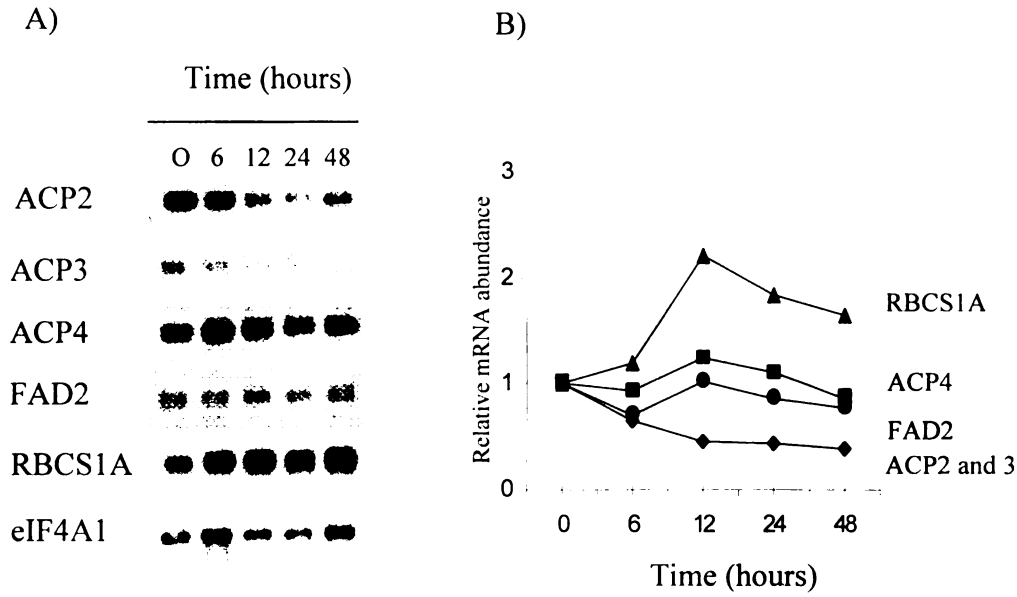

Figure 10. Differential regulation of ACP mRNA levels in starved Arabidopsis cells.

(A) Cells were starved for 48 hours in the presence of light and total RNA was extracted at the times indicated. Each lane of the Northern blots contains 2.5 μg of total RNA. The blots were hybridized with probes corresponding to the sequences indicated. The eIF4A probe was used as a loading control. (B) The signals in A were quantified by scanning densitometry and the values are represented as in Figure 5.

transcript levels. The same mRNA profile was observed for RBCS1A and similar mechanisms of regulation might be operating for both genes (Figure 11). In the case of FAD2 mRNAs, no differences in the relative levels of the corresponding messenger were found in the conditions tested (Figure 10 and 11). Thus, in contrast to ACP, neither starvation nor light altered FAD2 mRNA abundance in liquid cell culture.

To investigate whether sugars could have a direct role in the regulation of ACP transcript levels, we examined if ACP expression could be altered by uncoupling growth from the

presence of sugars in the media. For this purpose, cells were starved in an osmotic control media (58 mM mannitol) for 48 hours and subsequently transferred into media containing either 58 mM 3-O-methyl-glucopyranose (3-OMG) or 2 mM 2-deoxy-glucose (2-d-Glc). The effect of these two glucose analogs on gene expression has been previously studied in Arabidopsis cell-suspension cultures (Fujiki *et al.*, 2000). The first glucose analog is taken up by cells but not phosphorylated whereas the second is phosphorylated but not further metabolized (Dixon *et al.*, 1979). The presence of 3-OMG and 2-d-Glc in the media neither showed a positive effect on ACP2+3 transcript levels nor did it decrease the ACP4 mRNA level after 24 hours (data not shown). These results suggested that cell growth and/or a metabolic signal generated by sucrose or downstream of glucose were necessary to control ACP mRNA levels in Arabidopsis cell liquid culture.

ACP2 mRNA Levels are Affected by a Sucrose-derived Signal and/or Growth Control *in planta*

To evaluate if mechanisms that regulate ACP2 transcript levels in Arabidopsis cell cultures also operate in intact plants, Arabidopsis seedlings were grown in liquid media in the presence or absence of carbon. The results in Figure 12 indicated that ACP2 mRNA levels responded to carbon signals in intact plants similarly to cell suspension cultures. Therefore, the underlying mechanisms were most likely physiological mechanisms of regulation of ACP2 expression in plants rather than mechanisms acting only in cell suspension cultures.

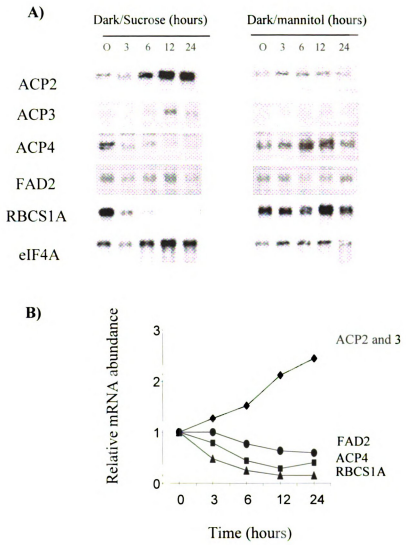

Figure 11. Differential regulation of ACP mRNA levels in Arabidopsis cells grown in the presence of sucrose.

(A) Cells were starved for 48 hours in the light and subsequently transferred to either sucrose containing media (dark/sucrose) or an osmotic control media (dark/mannitol) and incubated in the dark. Total RNA was extracted at the times indicated. Each lane of the Northern blots contains 2.5 µg of total RNA. The blots were hybridized with probes corresponding to the sequences indicated and the eIF4A probe was used as a loading control. (B) The blots corresponding to the dark/sucrose treatment were quantified by scanning densitometry and the values are represented as in Figure 5.

Transcriptional regulation of Arabidopsis *ACP2* gene by growth/cell cycle signals

The increase in ACP2 mRNA abundance after carbon induction (Figures 10, 11, 12) may be brought about by transcriptional or post-transcriptional mechanisms (e.g., mRNA stability).

Therefore, to investigate which mechanisms are involved in *ACP2* gene expression, a 1.5 Kbp DNA region upstream of this gene was fused to luciferase (LUC) reporter and transformed into Arabidopsis. In order to eliminate the effect on gene expression of the ACP2 5' leader (Figures 8 and 9), this region was not included in the construct (see materials and methods). As a control, a 2 Kbp DNA region upstream of the *eIF4A1* gene was fused to LUC. The steady-state levels of eIF4A1 mRNA remained constant regardless of the presence or absence of carbon in the media (Figures 10 and 11). In addition, all constructs carried a second reporter gene (GUS) under the constitutive CaMV35S promoter to normalize LUC activity for T-DNA copy number, position effect and in this case also for differential transcriptional activity in cells with variant energetic status (presence/absence of carbon sources).

Since Arabidopsis cells grown in liquid culture are recalcitrant to *Agrobacteria*-mediated transformation, transgenic cell suspension cultures were induced from transgenic Arabidopsis plants. Stable transformation was preferred for these experiments because problems associated with transient transformation (e.g., loss of vector and reporter gene activity) were avoided. This is particularly important when performing experiments that

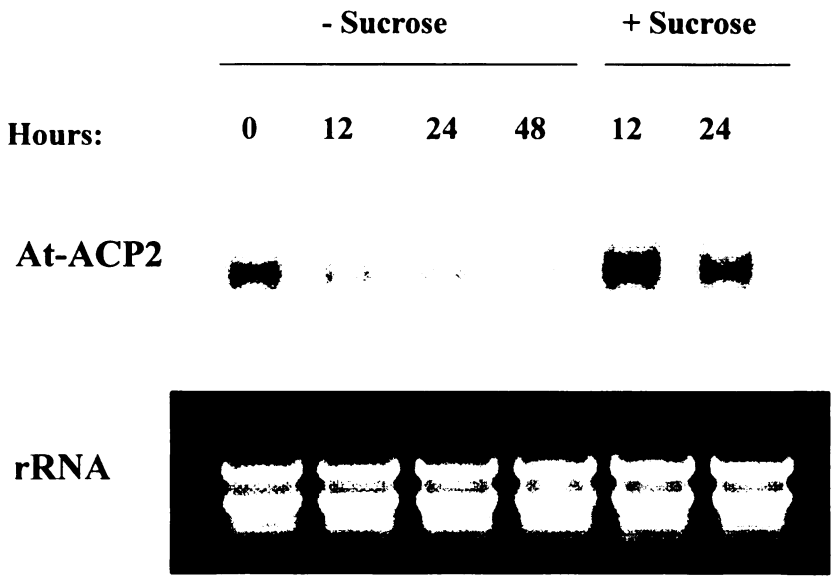

Figure 12. *In planta* regulation of ACP2 mRNA by carbon.

Arabidopsis seedlings were grown in liquid media for 2 weeks in the presence of 58 mM sucrose and light. Subsequently, seedling were transferred to media containing 58 mM mannitol and incubated for 2 days in the light (-Sucrose). Samples were taken at 0, 12, 24 and 48 hours and total RNA extracted. After 2 days of starvation seedlings were transferred to media containing 58 mM sucrose for 1 day (+Sucrose). Samples were taken at 12 and 24 h after induction and total RNA isolated. RNA was resolved by electrophoresis and blotted onto nylon membranes. Filters were hybridized with an Arabidopsis ACP2 cDNA probe (At-ACP2). Ribosomal RNA (rRNA) in the gel was visualized by ethidium bromide staining.

are carried out during several days. In addition, insertion of the transgene in the genome provides a better platform for regulation of ACP2 promoter activity. Interaction of foreign DNA with nucleosomes in the chromosome context should facilitate the organization of the promoter region (e.g., nucleosome position, binding of factors) to better reflect the actual promoter structure and regulation.

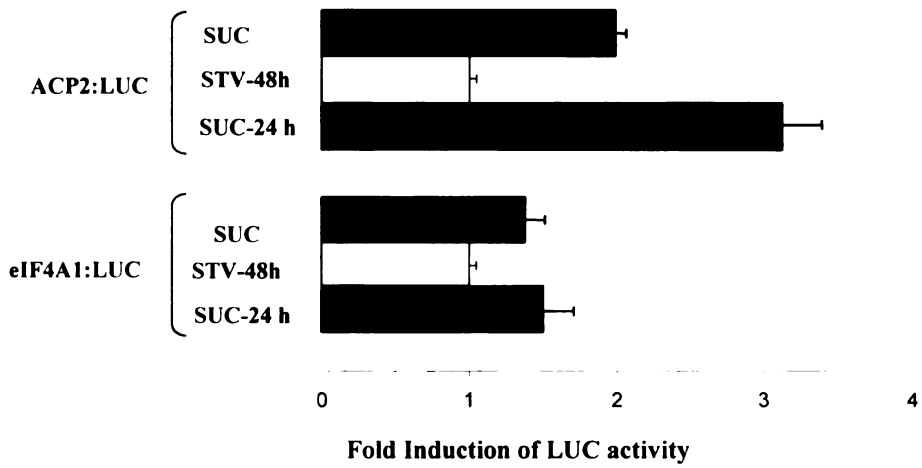

Figure 13. Regulation of Arabidopsis ACP2 promoter by carbon in Arabidopsis cell suspension culture.

Arabidopsis cell suspension cultures were generated from leaf tissue of transgenic plants carrying 1.5 Kb of ACP2 promoter (ACP2:LUC) and 2 kB of eIF4A1 promoter (eIF4A1:LUC). Vectors also carried the GUS reporter gene under a constitutive promoter (CaMV35S:GUS) to control for T-DNA position and copy number. Three independent cell liquid cultures for each construct were generated from three independent transgenic plants. Cells were maintained in media supplemented with 58 mM sucrose (SUC). At the beginning of the experiment, cells were starved in media containing 58 mM mannitol for 2 days (STV-48h). After this period cells were transfered to sucrose containing media for 1 day (SUC-24h). Cell samples were taken at the beginning and end of each period and LUC and GUS specific activities assayed in triplicate. LUC activity was normalized with GUS activity (LUC/GUS) and expressed as fold induction of LUC activity. LUC/GUS ratios were set to 1 for STV-48 to simplify the interpretation of the results.

The results in Figure 13 indicated that the LUC/GUS ratio of specific activities was reduced by 2-fold after 2 days of starvation and increased by more than 3-fold when sucrose was added back. Changes in LUC expression in transgenic Arabidopsis cells were similar to those in ACP2 mRNA levels under the same growth conditions (Figures 10 and 11). Moreover, no significant differences in LUC/GUS ratio of specific activities were observed when LUC was under regulation of eIF4A1 promoter. These results agreed with previous experiments in which eIF4A1 transcript levels did not change with carbon availability (Figures 10 and 11). Thus, a 1.5 Kb DNA fragment upstream of the *ACP2* gene promoter was sufficient to confer carbon regulation of gene expression. These results indicated that changes in ACP2 mRNA abundance were the result of transcriptional activation/de-repression by carbon rather than increased transcript stability.

Other carbon sources were tested for their ability to induce LUC expression in Arabidopsis cells. In this regard, glucose and fructose either alone or together were capable to activate LUC expression in cells carrying ACP2:LUC construct (Figure 14). These results demonstrated that metabolizable carbon sources other than sucrose were also capable to regulate *ACP2* gene promoter. To test if carbon sources different from sugars could also increase LUC expression, Arabidopsis cells were incubated with 3 mM sodium acetate after starvation. Acetate is rapidly taken up and used as substrate for fatty acid biosynthesis, however it is not a good source of carbon to sustain Arabidopsis cell growth (data not shown). Nevertheless, acetate regulates gene expression of genes

involved in various cellular processes (Sheen,1990). The results in Figure 14 indicated that acetate failed to induce LUC expression after starvation and suggested that growth

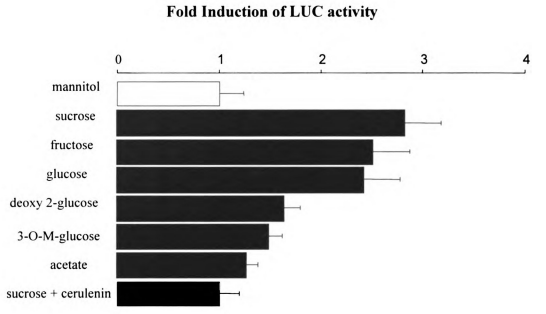

Figure 14. Regulation of ACP2 promoter by different carbon sources in Arabidopsis cell suspension cultures.

Transgenic Arabidopsis cells expressing ACP2:LUC and CaMV35S:GUS constructs were starved for 2 days in mannitol containing media and then induced with different carbon sources for 1 day. After this period, LUC and GUS specific activities were assayed in triplicate. LUC and GUS specific activities assayed in triplicate. LUC activity was normalized with GUS activity (LUC/GUS) and expressed as fold induction of LUC activity. LUC/GUS ratios were set to 1 for mannitol treatment to simplify the interpretation of the results. Sucrose, glucose and fructose were at 58 mM, 2-deoxy-glucose (2-deoxy glc) at 5 mM, 3-O-methyl glucose (3-O-methyl glc) at 58 mM, acetate at 3 mM, cerulenin at 0.01 mM . Bars denote standard deviations of the average.

and not the sole presence of carbon sources was necessary to regulate *ACP2* gene promoter.

To further investigate the relationships between ACP2 gene transcription, carbon supply and growth, two different strategies were followed. First, cells were incubated with sugar analogs (3-O-methyl-glucose and 2-deoxy-glucose) that are not metabolized by cells yet sensed as carbon and therefore provide a sugar-sensing signal (Fujiki *et al.*, 2000). Second, cells were incubated in the presence of sucrose plus 10 μM cerulenin. The rationale for the latter experiment was to provide carbon for growth but at the same time to halt growth/cell division by limiting supply of fatty acids. The results in Figure 14 indicated that both treatments, sugar analogs and sucrose plus cerulenin, failed to induce ACP2:LUC expression after starvation. These data suggested that growth and/or cell division is a requisite for regulation of transcription by the *ACP2* gene promoter. Possibly, transcription of *ACP2* gene may be regulated by cell-cycle signals. In agreement with this hypothesis, microarray profile analysis of synchronized Arabidopsis cell cultures demonstrated that ACP2 mRNA expression is cell-cycle coordinated (Menges *et al.*, 2002).

Deletion analysis of Arabidopsis *ACP2* promoter in tobacco BY-2 cells

To identify discrete DNA elements in the Arabidopsis ACP2 promoter responsible for transcriptional regulation by growth and/or cell cycle, a promoter deletion analysis was

conducted. A promoter deletion series of the Arabidopsis ACP2 promoter was fused to the luciferase reporter gene and the constructs used to transform tobacco BY-2 cells. Tobacco cells, instead of Arabidopsis, were chosen for this experiment because the former cells have the advantage of being transformable by Agrobacteria and consequently more constructs could be conveniently analyzed. Nonetheless, this system may have disadvantages. First, mechanisms existent in Arabidopsis to activate *ACP2* gene expression could be different from those in tobacco cells. Second, factors in tobacco cells may not recognize elements in the Arabidopsis ACP2 promoter.

The results in Figure 15 demonstrated that LUC expression driven by the 1.5 Kb ACP2 promoter responded to carbon signals in tobacco BY-2 cells, similar to Arabidopsis cells. Furthermore, the increase and decrease in LUC expression by carbon availability were in the same range as observed in Arabidopsis cells (Figure 13 and 14). Thus, these data indicated that mechanisms for *ACP2* gene expression were conserved between tobacco BY-2 cells and Arabidopsis cells and also that tobacco factors can recognize and regulate Arabidopsis ACP2 promoter by growth/cell cycle signals.

The deletion series of the ACP2 promoter disclosed the presence of both positive and negative domains for gene expression in the Arabidopsis ACP2 promoter (Figure 15). Removal of the –1,500/–750 fragment reduced LUC expression by 6-fold, indicating the presence of positive elements in this promoter area. Although overall LUC expression was affected, the response to carbon was unaltered, suggesting that growth/cell cycle responsive

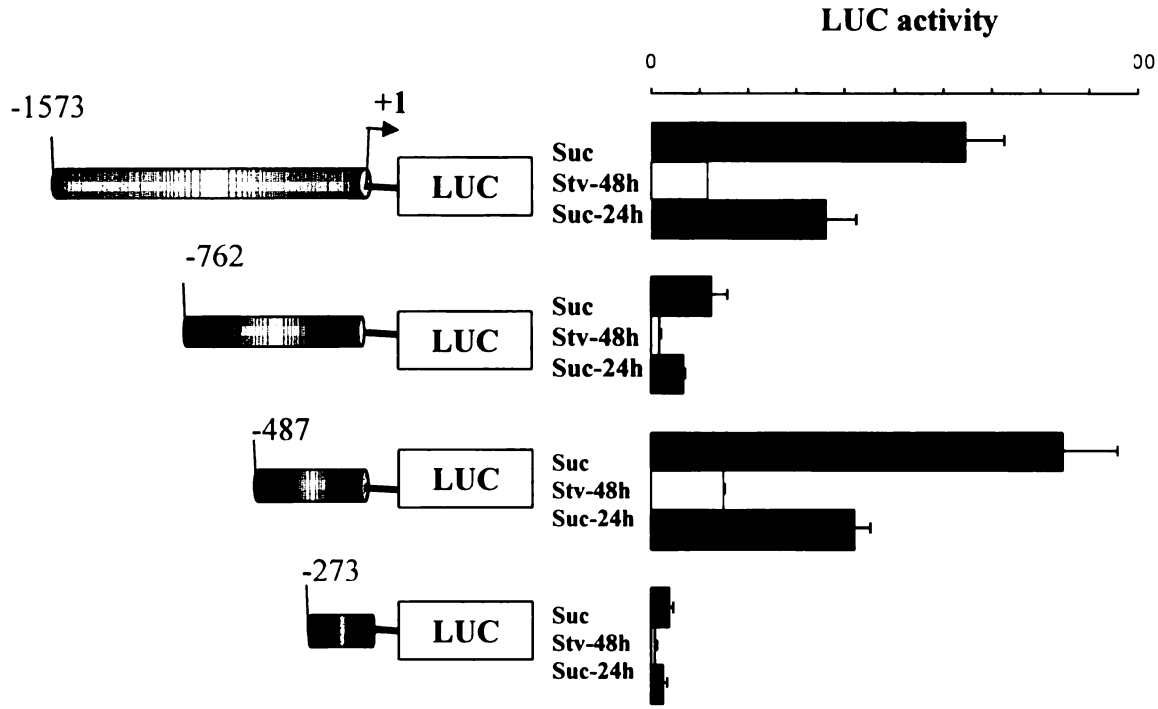

Figure 15. Arabidopsis ACP2 promoter deletion analysis in tobacco BY-2 cells.

Tobacco BY-2 cells were transformed via Agrobacteria with a binary vector containing a deletion series of the Arabidopsis ACP2 promoter fused to luciferase (LUC). Vector also carried GUS fused to CaMV35S. Three individual calli were used to generate three independent cell suspension cultures for each construct. Cells were maintained in media with 58 mM sucrose (Suc). At the beginning of the experiment cells were starved in media containing 58 mM mannitol for 2 days (Stv-48h). After this period cells were transferred to sucrose containing media for 1 day (Suc-24h). Cell samples were taken at the beginning and end of each period and LUC and GUS specific activities assayed in triplicate. Luc activity was normalized by GUS activity and expressed as LUC activity (units of light emitted).

elements were still present in the 750 bp promoter fragment. Interestingly, deletion of an additional 250 bp (-750 to –500) reconstituted LUC expression to levels similar to those obtained with the 1.5 Kb promoter fragment (Figure 15). This result indicated that

negative elements of expression were localized in the –750/-500 region. LUC expression still responded to carbon signals and therefore growth/cell cycle elements were present in the –500 promoter fragment. Further deletion of the promoter (-500 to –250) decreased by 10-fold the overall expression of LUC, indicating the presence of positive elements in the –500/-250 fragment. Induction of LUC activity by carbon was in the same range as with the other constructs (~ 2-3 fold) indicating the presence of growth/cell cycle responsive elements within the –250 promoter fragment (Figure 15).

Conserved elements in Arabidopsis *ACP* promoters

In addition to the conserved elements found in the 5' UTRs (Table 2), computer alignments of Arabidopsis *ACP* genes (plastidial isoforms) disclosed the presence of two additional conserved elements (Figure 16). First, an 11 bp element was present in the promoter regions of the five ACP genes (consensus sequence: CCTGCATCTCC). This element was neither present in ACP genes of other plant species nor in other Arabidopsis FAS and lipid biosynthetic genes (Beisson *et al.*, 2003). Therefore, it may be that the 11 bp element is specific for Arabidopsis ACP promoters. Nevertheless, it has to be considered that information on ACP promoter sequences from different plant species is still limited. Second, a 6 bp element (GCCAAA) was found at –200 bp in ACP2 and ACP3 promoter regions but not in the other Arabidopsis ACP promoters (Figure 16). Interestingly, the same element is present in FAS1 and FAS2 genes of yeast that are regulated by growth (Chirala, 1992; Schuller *et al.*, 1992) as well as in several Arabidopsis genes which are cell-cycle regulated (e.g., *At-CDC6, At-MCM3*) (Stevens *et*

Figure 16. Conserved elements in Arabidopsis ACP promoters.

Two conserved elements in the Arabidopsis ACP genes were identified by computer alignments. An 11 bp element (highlighted in gray) was found in the promoter regions of the five Arabidopsis ACP genes. The consensus sequence for this element is indicated at the bottom of the figure together with its location (relative to the start codon [capital letters]) in the different ACP genes. Interestingly, the 11 bp element in the ACP5 gene is located in the coding sequence. A 6 bp element GCCAAA (underlined) was found only in ACP2 and ACP3 promoters. This element overlaps with the 11 bp element and this observation may further suggest that these two elements are important for ACP gene expression.

ACP1

ttgtcCCTGGTTCTCCgactgagagaagcagccatgatcttagtaaaccttgaggagaaag atatatagaaacttaaccaaaaaacttcttcttgctcttccctcttatggtgactagtatt gtgtttcacgaaacaATG

ACP2

ttccaaCCTGCATCTGCCAAAgcacccaactccacctgacttgtctgtgtctgttgcgttca ctttcattggaatctcagatttttattttttgtttgttttggttgttgcgatttgtgactataaaacctctcc cacttggttcttcactctcactgtttctcatctcttcgtcttctccgcctccttcaatctcactccgatctc tctacgattcattcgttctATG

ACP3

tttgttatttctctaCCTGCATCTGCCAAA tcacccacctcacctgacttgtctgtctgaattct ctcattggaatctcagaaaagttttttttttttttttgtttaggttaaaatactataaaattaaaaataagtct cccacttggattcttcactgtgtctcacatctctttgtcttctccgcctcctccgatctcactccgatctc tctacgattcattcttc tATG

ACP4

gtgccgaagataggCCTGAATCTCCgagaacaaacctccgccacaaaaacagaagactt gttgcttgcgtatcataatacgacgccgtatctctacttgctcgaacaaacccaaaaagatacatat caagagattaaaccttatccaactaagagaagccatttttatttttttgggtctctgagttgtgtattga gcttcatctccttcaaATG

ACP5

ggatggtacttagaatagattttccaaaatgatggcagaatagaacgtggctctataaatacataaa tcccagcagtgtttgccatcagctacaaaatagtaattcacgctccttgaacaatccgccatctctct cgatcagatcgataATG gcgacaagtttctgcCCTCCATCTCC

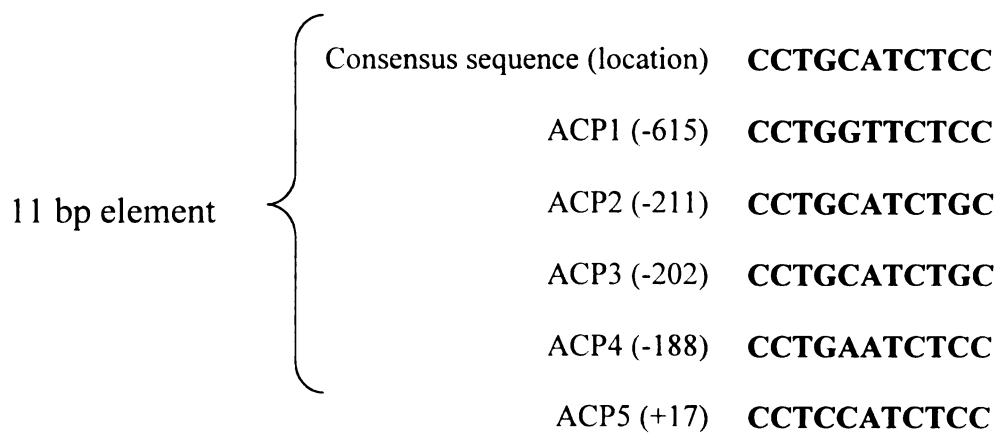

Figure 16

al., 2002; de Jager *et al.*, 2001).

Although ACP2 and ACP3 appear to derive from a recent gene duplication (these isoforms are > 80 % identical in their amino acid sequences and the respective genes lay one next to the other in the Arabidopsis genome), the divergent amino acid sequences of the other ACP isoforms (60-70 % identity) indicate that they diverged long ago. Therefore, the 11 bp element present in the promoter region of the five ACP isoforms represents a DNA motif that has been conserved for a long period throughout evolution. Thus, this element is a good candidate for a regulatory element of ACP expression. Because the presence of the 6 bp element in the ACP2 and ACP3 promoters is the result of a more "recent" gene duplication event, its conservation in these two promoters may only reflect its short existence in evolutionary terms. However, the presence of the same element in other Arabidopsis cell-cycle regulated genes suggests that the 6 bp element is also a good candidate for ACP gene regulation.

Changes in mRNA expression by inhibition of fatty acid synthesis in Arabidopsis cells

To investigate whether feedback mechanisms for FAS gene regulation similar to those in bacteria (Schujman *et al.*, 2003) also exist in plant cells, Affymetrix GeneChip analysis was used to follow changes in gene expression after inhibition of fatty acid synthesis in Arabidopsis cells. Gene expression analysis of cell suspension cultures presents several advantages over analysis of whole tissues. First, cell cultures are homogeneous systems as opposed to tissues which are composed of different cell types. Second, growth

conditions can be rapidly changed and cells more readily take up components from the media. Finally, cells share the same environment and therefore they should perceive similar signals. Cerulenin, a potent inhibitor of fatty acid synthesis in plants was utilized in the experiment. This drug crosses cell membranes rapidly and specifically inhibits condensing enzyme KAS I (β-ketoacyl-ACP synthase) (Schneider and Cassagne,1995).

In order to evaluate the concentration of cerulenin required to inhibit fatty acid synthesis significantly but with minimal cytotoxic effects, cells were incubated with 0, 10, 30 and 100 μM cerulenin for 0, 2 and 6 hours. Labeled acetate was added to the cultures and after the incubation period, cells were quenched with hot isopropanol and lipids extracted. Analysis of labeled fatty acids demonstrated that after 2 hours the inhibition of FAS was 65%, 82% and 94% for 10, 30 and 100 μM cerulenin, respectively, compared to the control (Figure 17). After 6 hours, inhibition of FAS in the presence of cerulenin remained similar to values measured at 2 hours (70%, 84% and 96% for 10, 30 and 100 μM cerulenin respectively) (Figure 17). These data indicated that the effect of cerulenin on FAS persisted after 6 hours of treatment. In addition, the constancy in the percentage of FAS inhibition throughout the experiment suggested no major cytotoxic effects by cerulenin on Arabidopsis cells during this period. Based on these results, a concentration of 10 μM of cerulenin was chosen for gene expression experiments. This low concentration of inhibitor is enough to substantially reduce fatty acid synthesis (70%) and yet provide small amounts of fatty acids that would minimize cytotoxic effects and stress responses.

FAS genes do not respond to inhibition of fatty acid biosynthesis in Arabidopsis cells

Affymetrix gene chips (8K) representing approximately one third of the Arabidopsis genome were used to evaluate changes in gene expression after inhibition of fatty acid synthesis. Two flasks with 100 mL of cells growing at exponential phase were incubated in the presence of 10 μM cerulenin for 6 hours. Samples (20 mL) were taken at 0, 2 and 6 hours after drug treatment from each of the flasks (two biological replications per time point). Total RNA was isolated from each sample and used to synthesize cDNA with poly(T) primers fused to the T7 promoter. Subsequently, T7:cDNAs were used as templates for cRNA synthesis by *in vitro* transcription in the presence of biotinylated-ATP. Finally, biotinylated-cRNA was used for GeneChip hybridization reactions.

The results in Table 3 indicated that mRNA levels from some genes involved in fatty acid and lipid metabolism changed slightly after 2 and 6 hours of inhibition of fatty acid synthesis. For example, the mRNA levels corresponding to the biotin containing subunit (BCCP) of acetyl-CoA carboxylase increased ~ 2-fold after 6 h of cerulenin treatment. A few other mRNAs showed slight increases (e.g., 1.4-fold for ACP2 and cytosolic acetyl-CoA carboxylase). The standard deviations of these values were within 5 % of the average value and therefore the fold increases were reliable. In contrast, the mRNA levels of other lipid related genes were reduced by cerulenin treatment. Transcripts for acyl-ACP

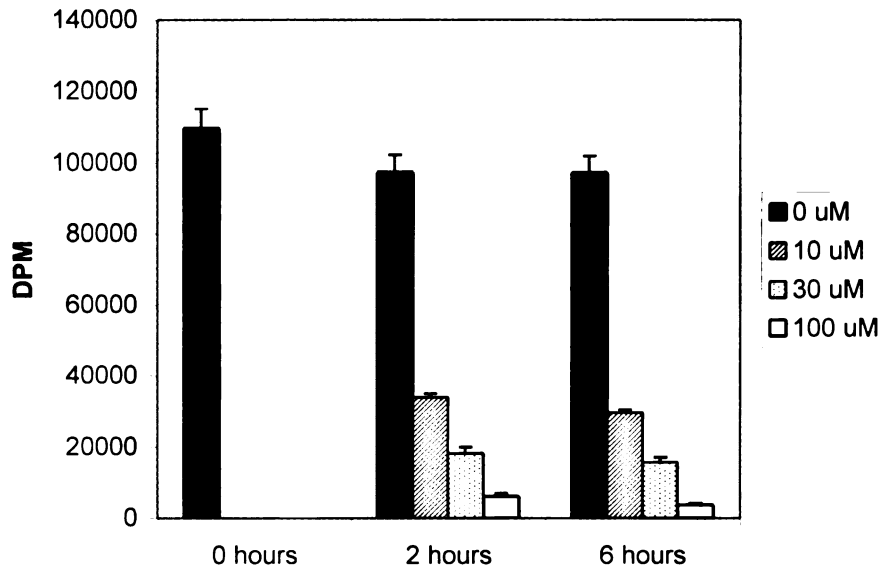

Figure 17. Inhibition of fatty acid synthesis by cerulenin in Arabidopsis cells.

Arabidopsis cells were incubated with 0, 10, 30 and 100 μM cerulenin for 0, 2 and 6 hours. Labeled acetate (0.025 mCi) was added to the cultures. After the incubation periods cells were quenched with hot isopropanol and lipids extracted. Radiolabeled fatty acids were transmethylated and separated by thin layer chromatography. Quantitation of bands corresponding to fatty acids was performed by scanning in an Instant-Imager and scintillation counting. Results are expressed in desintegrations per minute (DPM).

thioesterases declined approximately 1.6-fold after 6 h of drug treatment (Table 3). Thus, although small differences could be seen in the mRNA levels of some fatty acid synthesis genes, mRNA levels for most FAS and lipid synthesis genes were not significantly altered by inhibition of fatty acid biosynthesis.

Significant changes in mRNA levels were observed for genes related to cellular processes such as stress response, metabolism, drug resistance and several regulatory proteins such as transcription factors and kinases. Approximately 90 genes presented increases in transcript abundance of more than 2-fold after 6 h of cerulenin treatment (Table 4). Changes in the transcript levels of genes related to stress response, metabolism and drug resistance most likely reflected the stress conditions imposed by inhibition of fatty acid synthesis. Approximately 110 genes presented lower mRNAs levels than control cells after 6 h of cerulenin treatment (Table 5).

DISCUSSION

One objective addressed by the current work is the nature of the signals that give rise to changes in the expression of genes involved in fatty acid synthesis. Although a limited number of previous studies suggest a minor role of light on the expression of these genes (Battey *et al.*, 1990; Baerson *et al.*, 1993, Scherer *et al.*, 1987), the mutually dependent relation between chloroplast biogenesis and *de novo* production of glycerolipids envisage a closer connection between light and at least some of the genes for this pathway. In this report we demonstrated that light affects the expression of mRNAs encoding acyl carrier protein isoforms from Arabidopsis.

First, we demonstrated that the levels of the messenger for ACP4 increased 4- to 5-fold after light treatment of dark grown plants. The similarity in the kinetics of mRNA induction between ACP4 and FEDA in Arabidopsis suggests similar mechanisms of activation for both genes. Nevertheless, it remains to be examined whether the increase of

Table 3 . Changes in mRNA abundance of genes involved in lipid metabolism after inhibition of fatty acid synthesis in Arabidopsis cells.

	descriptions	Fold change	
		at 2 h	at 6 h
AT5G16390	acetyl-CoA carboxylase (BCCP subunit)	1.7	**2.1**
AT4G38570	putative phosphatidylinositol synthase	1.7	**2.1**
AT4G22340	CDP-diacylglycerol synthetase-like protein	1.5	**2.0**
AT1G36170	acetyl-CoA carboxylase subunit	1.2	1.5
AT1G54580	acyl carrier protein isoform 2	1.0	1.4
AT1G54580	acyl carrier protein isoform 2	1.2	1.4
AT3G51840	Short-chain acyl CoA oxidase	1.2	1.2
AT3G12120	delta-12 desaturase (Fad2)	-2.1	1.2
AT4G30950	omega-6 fatty acid desaturase (fad6)	1.0	1.2
DL4775C	thioesterase like protein	1.0	1.1
	glycerol-3-phosphate acyltransferase (ACT1)	1.0	1.1
AT1G01480	acetyl-CoA carboxylase (ACC2)	1.0	1.1
AT2G25710	biotin holocarboxylase synthetase	-1.1	1.1
AT5G05580	omega-3 fatty acid desaturase (fad8)	1.0	1.1
AT2G44620	acyl carrier protein precursor (mitochondrial)	-1.0	1.1
AT2G32260	putative phospholipid cytidylyltransferase	1.0	1.1
AT4G23850	acyl-CoA synthetase-like protein	1.3	1.1
AT1G67730	b-keto acyl reductase (glossy8)	1.3	1.1
AT4G13840	fatty acid elongase-like protein (cer2-like)	2.0	1.1
DL4770C	thioesterase like protein	1.6	1.1
AT5G35360	biotin carboxylase subunit (CAC2)	-1.3	1.1
AT4G20870	fatty acid hydroxylase-like protein	1.0	1.1
AT2G31360	delta 9 desaturase	1.0	1.1
AT2G33150	3-ketoacyl-CoA thiolase	-1.3	1.1
AT3G11170	omega-3 fatty acid desaturase	1.0	1.1
AT4G34510	putative ketoacyl-CoA synthase	-1.3	1.1
AT1G76490	3-hydroxy-3-methylglutaryl CoA reductase	-8.3	1.1
AT3G11170	fatty acid desaturase	-1.9	-1.1
AT4G34250	fatty acid elongase-like protein	-1.0	-1.1
DL4435W	triacylglycerol lipase like protein	-1.2	-1.1
AT1G68530	very-long-chain fatty acid condensing enzyme CUT1	-1.0	-1.1
AT4G25050	acyl carrier-like protein (ACP-4)	1.0	-1.2

***Fold change: fold increase in signal intensity compared to time o h**

Table 4. Example of transcripts that increase more than 2 fold after 6 hours of cerulenin treatment.

	descriptions	Fold change	
		at 2 h	at 6 h
T02O04.8	jasmonate inducible protein isolog	9.0	11.2
AT5G13930	chalcone synthase (CHS)	1.9	10.8
AT2G34660	multidrug resistance-associated protein 2	7.5	9.3
AT4G19030	nodulin 26 like protein	14.2	8.7
AT4G12360	putative lipid transfer protein	6.5	5.4
AT1G17740	Phosphoglycerate dehydrogenase	2.6	5.3
AT1G14900	high mobility group protein a (HMGa)	1.9	5.1
	AtMYB42 R2R3-MYB transcription factor.	9.0	5.0
AT2G33810	squamosa promoter binding protein-like 3.	4.5	4.7
AT5G18170	glutamate dehydrogenase 1 (GDH1)	2.4	4.6
AT1G10460	germin-like protein (GLP7)	4.7	4.6
AT4G21880	membrane-associated salt-inducible protein	4.0	4.6
AT5G08640	flavonol synthase	1.6	4.2
AT2G16590	putative protein	24.7	4.1
AT3G22490	LEA D34 protein homologue type1.	2.6	4.0
AT1G78820	receptor like ser/threo kinase ARK3	3.7	4.0
AT2G47000	multi drug resistance proteins	4.4	3.8
AT2G01830	putative histidine kinase (CRE1)	3.0	3.6
F13P17.32	cytochrome P450 homolog	2.4	3.5
F16N3.5	prolamin box binding factor (PBF)	3.7	3.4
AT1G09530	phytochrome-associated protein 3 (PAP3).	2.0	3.3
AT1G19050	ARR7 mRNA for response regulator 7	3.8	3.2
AT2G11620	putative	3.3	3.2
AT2G18650	RING zinc finger protein	2.8	3.1

***Fold change: fold increase in signal intensity compared to time o h**

Table 5. Example of transcripts that decrease more than 2-fold after 6 hours of cerulenin treatment.

	descriptions	Fold change	
		at 2 h	at 6 h
AT5G57560	xyloglucan endotransglycosylase (TCH4)	-3.45	-8.85
AT4G27280	calcineurin B-like protein 3	-4.05	-8.45
AT3G47380	putative	-2.35	-6.95
AT2G19800	putative	-0.55	-6.40
AT1G32170	xyloglucan endotransglycosylase-related protein	-3.55	-6.35
AT4G27450	glutamine-dependent asparagine synthetase	-1.20	-5.75
	late embryogenesis abundant protein homolog	-2.90	-5.65
AT2G37170	aquaporin	-4.50	-5.40
AT4G23550	putative protein	-1.60	-5.40
AT3G47340	glutamine-dependent asparagine synthetase	-3.55	-5.40
AT2G33830	putative auxin-repressed protein	-3.25	-5.30
AT1G13110	cytochrome P450	-0.15	-5.20
AT2G44380	putative CHP-rich zinc finger protein	-0.40	-5.05
AT2G28630	putative fatty acid elongase	-1.60	-5.00
	class 1 non-symbiotic hemoglobin	-1.15	-5.00
AT5G59520	zinc transporter (ZIP2)	-4.50	-4.95
AT4G02380	putative	-2.80	-4.95
AT5G24090	acidic endochitinase gene	-3.55	-4.95
	class IV chitinase	-3.70	-4.90
AT3G57700	protein kinase-like protein	-3.85	-4.85
DL4265W	membrane transporter like protein	-5.15	-4.80
A_TM017A05.3	putative	-0.75	-4.80
AT2G44840	ethylene response element binding protein	-4.50	-4.75
AT4G08950	putative phi-1-like phosphate-induced protein	-2.20	-4.65
AT4G22590	trehalose-6-phosphate phosphatase-like protein	-2.50	-4.65

***Fold change: fold decreased in signal intensity compared to time o h**

the ACP4 transcript levels is direct due to light (e.g. via phytochromes) or indirect via cell growth. The analyses of 1 Kb of genomic sequence upstream of the *Acl1.4* gene disclosed the presence of several GATA-like motifs. The GATA (or I) boxes are regulatory elements that are functionally important in many light-regulated promoters. The core element is defined as GATAA and related GATA motifs with variable flanking sequences are found in several promoters (Terzaghi *et al.*, 1995). For instance, the 1Kb upstream region of the ferrredoxin-A gene contains seven copies of related GATAA elements. Although the actual transcription initiation site for the ACP4 gene has not been mapped yet, we localized the GATA-like motifs respective to the AUG initiation codon of ACP4. Thus, the upstream region of this gene presents two AAGATAA elements at –715 and -812, one AGATAA at –505 and three GATAA at –70, -526 and –908 base pairs respective to the translation initiation codon. The presence of these elements in the upstream region of the gene for ACP4 suggests the direct role of light on the transcription of this gene. In contrast, only one and two copies (expected by chance) of these elements are present in the upstream regions (1 Kb) of *Acl1.2* and *Acl1.3* genes respectively. This observation is consistent with results that demonstrate that light has no impact on mRNA levels of ACP2 and most likely ACP3 (Figure 5 and Baerson *et al.*, 1993).

A second aspect of the influence of light on ACP gene expression was observed at the level of ribosome association. A general effect of light on polysome formation has been previously described in several studies (Giles *et al.*, 1977; Mosinger *et al.*, 1983). These works show that the proportion of ribosomes present as polyribosomes increases substantially in response to light. Moreover, continuous far-red (FR) light mediates a

strong increase in the relative level of polysomes, demonstrating the participation of phytochromes in the response. Despite this general effect on translation, there is evidence that particular mRNAs are less affected by the decrease in the translation rate in the absence of light. For example, Berry *et al* (1990) showed that in Amaranth cotyledons the expression of the small and large subunits of Rubisco are not found associated with polyribosomes in cotyledons of dark grown plants but rapidly recruited onto them upon illumination. In contrast, mRNAs encoding non-light regulated proteins are associated with polysomes regardless of the light-dark treatment. Our results (Figure 6) demonstrated that the transcripts for the ACP isoforms tested and FEDA largely dissociated from polysomes during the dark period in contrast to the non-plastidic eIF4A mRNA, which remained associated with high molecular weight polyribosomes in the dark. Thus, these observations reinforce the notion that cytoplasmic mRNAs are differentially associated with polyribosomes during the light-dark period (Berry *et al.*, 1990; Petracek *et al.*, 1997).

In chloroplasts of *Chlamydomones* and plants, both translation and mRNA stability are enhanced by nuclear encoded factors that bind to the 5' UTR of several chloroplast-encoded RNAs (Danon *et al.*, 1991; McCormac *et al.*, 2000). Likewise, several studies confirmed the major role of the 5'UTR in translation and mRNA stability of cytoplasmic mRNAs (Dickey *et al.*, 1998; Lukaszewicz *et al.*, 1998). A previous report demonstrated that these regions can also be important for transcription activation (Bolle *et al.*, 1994 and 1996). Analyses of the 5' leader sequences of several genes encoding for thylakoid proteins in spinach, disclosed the presence of CT-rich regions, designated CT-leader

boxes (CT-LB and CT-B) (Bolle *et al.*, 1994). The deletion of these elements from the PsaF (subunit III of photosystem I) and PetH (plastocyanin) upstream gene sequences severely reduces the transcription of a reporter gene in transgenic tobacco (Bolle *et al.*, 1994). It is noteworthy that the CT-LB box is also present in the 5'UTR of the spinach ACP II (Bolle *et al.*, 1996). The presence of several CT-rich elements and the CTCCGCC box in the ACP 5'UTRs of several diverse plant species (Table 2) suggests that these motifs might be important for ACP gene expression. The results presented in Figure 9 provide convincing evidence that these regions have a positive effect on reporter gene expression in transgenic Arabidopsis plants. Moreover, this effect was enhanced by light, suggesting that the ACP 5'UTRs may contain light-responsive elements that increase gene expression post-transcriptionally and/or transcriptionally in the context of the CaMV35S promoter (Figure 9). We also demonstrated that the 5' UTRs of Arabidopsis ACP1 and ACP2 conferred differential reporter gene expression in a tissue specific manner (Figure 9). At this point it is not possible to distinguish between a transcriptional, translational or mRNA stability mechanism to explain these differences in reporter gene activity.

Gallie *et al* (1992) showed that 5' leader length alters the expression of a reporter gene in carrot protoplasts. In particular, translation is influenced by leader length, and a 74 base leader construct is expressed 6-fold more highly than a 29 base leader construct. Conversely, Bolle *et al* (1994) found that in transgenic tobacco, the expression of a reporter gene under CaMV35S promoter is not altered when the 5' leader of PsaF (188 bases) is added to the 5' UTR (24 bases) of the native reporter gene mRNA. Based on

these results, it is possible that part of the differences observed in the LUC/GUS ratios in transgenic Arabidopsis tissues could be attributed to differences in the length of the ACP and control leaders (Figure 8 and 9). However, the tissue specific differences in reporter gene activity conferred by the three constructs analyzed, that ranged from almost no effect in roots to 10 to 30-fold increase in leaves and seeds, supports a sequence-dependent enhancement by the ACP 5' UTRs.

The results presented in Figures 10 and 11 for Arabidopsis mesophyll-derived cell culture indicate that the messengers for ACP2, ACP3 and ACP4 are under growth and/or metabolic control. The induction of the ACP2 and ACP3 transcript levels in the presence of 58 mM sucrose together with the inability of sugar analogs to reproduce this effect demonstrates that cell growth and/or a signal generated by sucrose or downstream of glucose is required for up-regulation of these messenger RNAs. In contrast to the response of these messenger RNAs, the ACP4 mRNA levels remained steady in starved cells and in the presence of 3-O-methyl-glucopyranose whereas they were down-regulated in the presence of 58 mM sucrose in the dark (Figure 10 and 11). The observation that mRNA levels for the ER localized FAD2 desaturase showed no variation under the conditions analyzed indicates that the response of genes encoding plastid components of fatty acid synthesis may differ from those controlling later steps such as cytosolic desaturation.

Regulatory mechanisms of *ACP2* gene expression also responded to carbon signals *in planta,* suggesting that these mechanisms were physiologically relevant and not the

consequence of cell culture induction. Analysis of the ACP2 promoter demonstrated that carbon-mediated regulation of *ACP2* gene expression was transcriptional and that promoter elements responsible for this regulation were located within –250 bp from the transcription initiation site. Comparison of Arabidopsis ACP promoters identified a GCCAAA element located at –200 bp of *ACP2* and *3* genes. This element was also found in yeast FAS genes (Chirala, 1992; Schuller *et al.*, 1992) and Arabidopis cell-cycle regulated genes (Stevens *et al.*, 2002; de Jager *et al.*, 2001). Therefore, it is a good candidate element for growth/cell cycle regulation of *ACP2* gene expression.

Analysis of gene expression after inhibition of fatty acid synthesis suggested that, in contrast to bacteria, fatty acid and lipid synthesis genes in plants did not respond to feedback mechanisms of gene expression after blocking this primary pathway. Thus, mechanisms other than regulation of FAS mRNA abundance may be involved in counteracting the inhibitory effect of cerulenin in plant cells (e.g., post-translational modifications of gene expression, metabolic regulation of FAS enzymes).

In summary, results of this study demonstrate that expression of genes involved in plant fatty acid synthesis is under multiple levels of control. Light clearly has a major impact on ACP4 mRNA levels, but this impact does not extend to the other ACP isoforms examined here. These different responses to light on ACP are probably transcriptional, based on the presence of GATA boxes in the ACP4 but not other ACP upstream regions. Light control is also exerted post-trancriptionally because all ACP isoforms are more highly associated with polysomes in the light than the dark. Experiments with suspension

cultures indicate that differences in expression patterns of ACP isoforms are also observed in their response to sugar supplements. A common element in both the leaf and tissue culture experiments is that ACP4, the most leaf-specific isoform, behaves in a manner similar to mRNA for genes involved in photosynthesis (FEDA or RBCS1A). In contrast, expression of the other ACP isoforms may be most responsive to demands for fatty acid synthesis brought about by enhanced growth. This level of complexity demonstrated by multiple ACP genes under different controls may reflect the need of plant cells to tightly regulate both the amount and the cellular destination of fatty acids produced in plastids (Ohlrogge and Browse, 1995) and to match the supply of fatty acids to different tissue and environmental demands.

MATERIALS AND METHODS

Nomenclature

There are five genes for plastidial ACP in the *Arabidopsis thaliana* genome (Mekhedov *et al.*, 2000). In this study we report results for *Acl1.1*, *Acl1.2*, *Acl1.*3 and *Acl1.4* genes, corresponding to the gene products ACP1, 2, 3 and 4 respectively. *Acl1.1* (GenBank accession number X13708) was previously described in Post-Beittenmiller *et al.*, 1989 and *Acl12.2* and *Acl1.3* (GenBank accession numbers X57698 and X57699 respectively) in Lamppa *et al.*, 1991. We propose that the ACP major leaf isoform (Shintani, 1996) gene be named *Acl1.4* (isoform ACP4, At4g25050 or F13M23.190) and that *Acl1.5* (ACP5) referred to GenBank accession number: A_TM021B04.6 or AF007271.

Plant Growth and Lighting conditions

Wild type *Arabidopsis thaliana* plants (ecotype Columbia) were grown on soil at 20°C in a 12:12 hours light:dark photoperiod (80-100 μmol m^{-2} s^{-1}). For the analyses of light-mediated induction of mRNA levels, 2 week-old plants were left in the dark for 24 hours and reilluminated with white fluorescent light (80-100 μmol m^{-2} s^{-1}) for the times indicated in Figure 5. Leaf tissue was harvested and immediately frozen in liquid nitrogen prior to RNA extraction. For polyribosomal association analyses, 2 week-old plants were dark incubated for 12 hours and then incubated in the dark for an additional 12 hours or illuminated for 12 hours. Leaf tissue was harvested at the end of each period and processed as indicated above.

Constructs and Plant Transformation

Sequences corresponding to the 5' untranslated regions of ACP1 (GenBank accession number X13708) and ACP2 (X57698) mRNAs were obtained by PCR amplification of Arabidopsis (ecotype Columbia) genomic DNA using the primers: 5'CATGCTCGAGCTCTTTGTACACTCCGCCCT3' and 5'-CATGCCATGGCGCTGAATTGAGTCGCCAT-3' for ACP1 (gene *Acl1.1*); 5'-CATGCTCGAGACTGTTTCTCTATCTCTTTG-3' and 5'-CGCGCCATGGCAATGGAAGCCATAGAAGAAT-3' for ACP2 (gene *Acl1.2*). The PCR amplification products were digested with *Xho*I and *Nco*I and cloned in frame to the

firefly luciferase gene (LUC) in pUC-LUC-BT2 (Weißhaar *et al.*, 1991) to give pTACP1 and pTAC2 respectively. A truncated version of the ACP1 5' leader sequence was generated by *in vitro* annealing of two synthetic oligonucleotides: 5'-TCGAGCTCTTTGTACAAACAATGGCGACTCAATTCAGCGC-3' and 5'-CATGGCGCTGAATTGAGTCGCCATTGTTTGTACAAAGAGC-3'. The double-stranded fragment was cloned in frame to the LUC coding region in pUC-LUC-BT2 to give pTdel1. All constructs were confirmed by sequencing. The vector pTACP1 was digested with *BamH*I and the vectors pTACP2 and pTdel1 with *Bgl*II. The resulting fragments were cloned independently in the *BamH*I site of the binary vector pCAMBIA-2201 (Hajdukiewicz *et al.*, 1994) to generate pCaACP1, pCaACP2 and pCadel1 respectively (Figure 8). The orientation of the inserts was confirmed by restriction mapping using PstI. *Agrobacterium tumefaciens* strain C58C1 (pM90) (Koncz *et al.*, 1986) was transformed with the pCaACP1, pCaACP2 and pCadel1 binary vectors by the freeze/thaw transformation protocol (An, 1987). The leaf-vacuum infiltration method was used for Arabidopsis transformation (Bechtold *et al.*, 1993). The transgenic lines transformed with pCaACP1, pCaACP2 and pCadel1 were named atL-acp1, atL-acp2 and atL-del1 respectively.

A 1.5 Kb DNA fragment upstream of the Arabidopsis ACP2 gene was amplified by PCR using the following primers: fwd, 5' GACTGGATCCTGCATTAGCTGGTAGTTCAG 3' and rvs, 5' GACTCTCGAGTGAGAAACAGTGAGAGTGAA 3'. The resulting PCR fragment was digested with *BamH*I and *Xho*I and ligated into the pUC-LUC-BT2 vector upstream of the luciferase coding sequence (Td-2-ACP2-LUC). A 2 Kbp upstream region

corresponding to the Arabidopsis *eIF4A1* gene was amplified by PCR using the following primers: fwd, 5' GACTGGATCCGAAAACGAACCTCAGTTCAG 3' rvs, 5' GACTCTCGAGGAGAGACTGGTGGAATATCC 3'. The resulting PCR fragment was digested with *BamH*I and *Xho*I and ligated into the pUC-LUC-BT2 vector upstream of the luciferase coding sequence (Td-2-eIF4A1-LUC). The constructs were digested with *Bgl*II and independently subcloned into the *BamH*I site of pCAMBIA-2201 binary vector. The orientation of the insert was analyzed by digestion with Pst-I and sequencing.

For construction of the ACP2 promoter deletion series, the vector Td-2-ACP2-LUC carrying 1.5 Kbp upstream region of the ACP2 gene was used as template for PCR. A combination of forward primers with the same reverse primer (same sequence as rvs primer described above) was used to generate the deletions. The forward primers were as follows: D750, 5' GACTGGATCCGTAGTA AACCACTATTGAAA 3'; D500 5' GACTGGATCCCACATAGTAAATTGATGCGT 3'; D250 5' GACTGGATCCGACGTAGTAGAATTTACAAA 3'; D100 5' GACTGGATCCTGACTTGTCTGTGTCTGTTG 3'. The PCR products were digested with *BamH*I and *Xho*I and ligated into Td-2 vector. The different constructs were digested with *Bgl*II and ligated into the *BamH*I site of pCAMBIA 2201 as indicated above. All constructs were confirmed by restriction mapping and sequencing.

Selection of Transgenic Plants and Tissue Collection

Seeds (T1) from transformed Arabidopsis plants were surface-sterilized and sown on seed germination media in the presence of 50 μg/mL kanamycin. After one week, resistant seedlings (T1 plants) were transplanted individually to soil and grown at 20°C in a 18:6 hours light:dark period until they set seeds. For reporter gene analyses in leaf tissue, seeds from T2 plants were selected in germination media under 50 μg/mL kanamycin and transferred to soil. Plants from 10 independent transgenic lines for each construct (see Figure 9) were grown in a 12:12 hours light:dark cycle for 2 weeks. The last day the plants were kept in the dark for 24 hours and the leaf tissue from half of the plants per line was collected and immediately frozen in liquid nitrogen for subsequent enzyme analyses. Leaf tissue from the remaining plants was harvested as above after 6 h of reillumination with white fluorescent light (80-100 μmol m^{-2} s^{-1}). For reporter gene analyses in developing seeds, T2 seedlings were selected as above and transferred to soil. Ten independent lines per construct (see Figure 9) were grown in a 16:8 hours light:dark cycle. Flowers from the primary stem were tagged in the morning every day during one week. Flower anthesis was considered as day zero in our experiment, and mid-stage siliques (8 to 10 days after anthesis) were dissected and the seeds immediately frozen in liquid nitrogen for subsequent enzyme analyses.

Cell Suspension and Root Liquid Cultures

Arabidopsis (ecotype Columbia) suspension cultures (Axelos *et al.*, 1992) were a gift from N. Raikhel laboratory (Michigan State University, DOE Plant Research Laboratory). Suspension cultures were maintained in a 12:12 hour light:dark photoperiod

at 22°C on a rotary shaker (120 rpm) in CSM-suc media (0.32% (w/v) Gamborgs with minimal organics (Sigma G5893), 58 mM sucrose, 0.05% (w/v) MES, 1.1 μg mL^{-1} 2,4D, pH 5.7). For the analyses of mRNA levels in different growth conditions, 100 mL cell cultures were grown for 3 days in CSM-suc in the presence of white light (100 μmol m^{-2} s^{-1}). After the third day, the cells were pelleted at 700 rpm and washed twice with CSM-ma (0.32% (w/v) Gamborgs with minimal organics (Sigma G5893), 58 mM mannitol, 0.05% (w/v) MES, 1.1 μg mL^{-1} 2,4D; pH 5.7). Cells were finally resuspended in 150 mL of CSM-ma and incubated in the presence of light for 2 days (starved cells). An aliquot of 10 mL was taken at the times indicated in Figure 10 and the cell pellet stored at -80°C until RNA extraction. The remaining 100 mL of the culture was pelleted as before, resuspended in either 50 mL of CSM-suc or 50 mL of CSM-ma and incubated in the dark for 24 hours. An aliquot of 10 mL was taken at the times indicated in Figure 11 and the cell pellet stored at -80°C until RNA extraction. In the experiment with glucose analogs, cells were starved with CSM-man for 2 days and subsequently transferred to CSM supplemented with either 58 mM of 3-O-methyl-D-glucopyranose (Sigma) or 0.2 mM of 2-deoxy-D-glucose (Sigma). Cells were incubated in the dark and an aliquot of 10 mL was taken at 0, 3, 6, 12 and 24 hours and the cell pellet stored at -80°C until RNA extraction.

Induction of Arabidopsis cell suspension culture from leaf tissue was performed as described by Axelos *et al* (1992). Tobacco bright yellow-2 cells (BY-2) were transformed via *Agrobacterium tumefaciens* strain LBA4404 as described by Bao *et al* (2002).

For root liquid culture, approximately 50 seeds from ten independent transgenic lines (T2 plants) per construct (see legend Figure 9) were surface sterilized and incubated in root liquid media (0.43% (w/v) MS salts, 0.05% (w/v), MES, 0.1% (v/v) 1000X B5 vitamin stock, 50 μg mL^{-1} kanamycin, pH 5.7) at 4°C for two days. The seeds were then incubated at 22°C in continuous fluorescent white light on a rotary shaker (120 rpm) for 2 weeks. Root tissue from approximately 20 seedlings per transgenic line was collected under the dissecting microscope and frozen in liquid nitrogen for subsequent enzyme analyses.

RNA Extraction and Northern Analyses

Total RNA was isolated from plant tissue and cell pellets by standard phenol/chloroform extraction and lithium chloride precipitation (Sambrook *et al.*, 1989). The RNA was fractionated in formaldehyde-agarose gels and blotted onto Hybond-N filters (Amersham-Pharmacia Biotech, NJ). In all cases hybridization reactions were done at 42°C in 50% formamide with α-[^{32}P]-dCTP radiolabeled fragments corresponding to ACP2 (X57698), ACP3 (X57699), ACP4 (RXW18), FEDA (M35868), eIF4A (X65052), FAD2 (L26296) and RBCS1A (X13611). After autoradiography, the relative mRNA levels from the northern blots were quantified by scanning densitometry (Molecular Dynamics). Messenger specific probes for ACP2 and ACP3 transcripts were generated by PCR amplification of Arabidopsis (ecotype Columbia) genomic DNA corresponding to the 3'UTR of these transcripts. The primers used were: 5'-TGAAAAGGCCAAGTAGAAT-3' and 5'-GTCAGATACAAGCCTTGTA-3' for

ACP2; 5'-GGAAAAGGCCAAGTAGAAA-3' and 5'-CAAGCCTTGTAATAATTATC-3' for ACP3.

Polyribosome Analyses

The protocol for polyribosome isolation was adapted from Petracek *et al* (1997) with the following modifications: approximately 1 g of *Arabidopsis* leaf tissue or *B.napus* developing seeds were homogenized in 4 mL of U buffer (200 mM Tris-HCl , pH 8.5, 50 mM potassium chloride, 25 mM magnesium chloride, 2 mM EGTA, 100 μg/mL heparin, 2% polyoxyethylene, and 1% deoxycholic acid), centrifuged at 13,000 *g* for 15 min at 4°C and the complete supernatant (4 mL) was loaded onto a 30 mL linear sucrose gradient (15 to 60%). After gradient centrifugation, fifteen 2 mL fractions were collected by dripping directly into 2 mL of phenol-chloroform, 50 μL of 10% SDS, 40 μL of 0.5M EDTA, and 10 μL of 100 mM aurin-tricarboxylic acid (Sigma). The final RNA pellets were washed with 70% ethanol, dried and resuspended in 10 μL water, 20 μL formamide, 6.5 μL formaldehyde, and 3.5 μL MOPS buffer (0.2 M MOPS, pH 7, 50 mM sodium acetate, 5 mM EDTA). Samples were heated for 15 min at 68°C and loaded onto a formaldehyde-agarose gel. Northern analyses were performed as described above. For gradient UV profile analyses, two gradients were spun in parallel and one used to measure UV absorbance. Fifty 100 μL samples were taken at identical volume intervals and the absorbance measured at 260 nm.

Tissue Extraction and Enzyme Assays in Transgenic Plants and Cells.

Leaf tissue or cell pellets (~ 0.25 g) were ground with a mortar and pestle and resuspended in either 300 µL of GUS buffer (200 mM Trizma-HCL, pH 7.0, 10 mM ß-mercaptoethanol, 10 mM EDTA, 0.01% (w/v) SDS, and 0.1% (v/v) Triton X-100) or 300 µL of LUC buffer (100 mM potassium phosphate, pH 7.8, 1 mM EDTA, 7 mM ß-mercaptoethanol, 10 % (v/v) glycerol) and further homogenized on ice. Root tissue (~ 0.2 g) was homogenized and resuspended in 100µL of either GUS buffer or LUC buffer. Developing seeds (~ 0.1 g) were homogenized in either 200 µL of GUS buffer or LUC buffer. In all cases, cell debris was removed by centrifugation for 15 min at 13,000*g* at 4°C. The supernatant was transferred to a clean tube and kept on ice. For GUS activity, 50 µL of the sample supernatant were diluted in 400 µL of GUS buffer and mixed with 50 µL of 10 mM 4-methylumbelliferyl-b-D-glucuronide (Sigma). The reaction was incubated at 37°C and a 100 µL aliquot was diluted in 0.2 M Na_2CO_3 at time 0 and every 15 min for 60 minutes. UV fluorescence was measured at 365-nm excitation and 455-nm emission with a Hitachi F-2000 fluorometer. GUS specific activity is expressed as nmol methylumbelliferone produced min^{-1} mg^{-1} protein. For LUC analyses, a 25 µL aliquot of the sample was mixed with 100 µL of LUC reagent (20 mM Tricine, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 270 µM CoA, 500 µM luciferin (Promega), 500 µM ATP). The linear range of the reaction was determined by mixing increasing extract volumes with 100 µL of LUC reagent. Luminescence was measured (3 s delay, 10 s integration time) in a Turner TD-20e luminometer. LUC specific activity is expressed as light units/mg protein. In all cases the GUS and LUC specific activities and LUC/GUS ratios were measured in triplicate and independently for each transgenic line. Protein

concentration was determined using a protein assay kit (Bio-Rad) with BSA (Sigma) as the standard.

Determination of FAS inhibition by cerulenin in Arabidopsis cell cultures.

Cells were incubated in the presence of 0, 10, 30 and 100 μM of cerulenin and 25 μCi of [1-^{14}C] sodium acetate for 0, 2 and 6 hours. Total lipids were extracted by hexane-isopropanol method and fatty acids transmethylated with 5% (v/v) sulfuric acid in methanol for 1 hour at 80° C. FAMES were separated by thin layer chromatography (TLC) with 90/10 (v/v) hexane/diethyl-ether. Radiolabeled bands corresponding to fatty acids were quantitated by scanning in an Instant Imager (Packard, Meriden, CT) and scintillation counting (Beckman Instruments, Fullerton, CA).

Affymetrix GeneChip analysis.

All the experimental protocols were performed according to the Affymetrix GeneChip® Expression Analysis Manual (http://www.affymetrix.com). Data analysis was performed with GeneChip® Data Mining Tool Software (Affymetrix, Inc; Santa Clara, CA).

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CHAP TER 3

DISRUPTION OF THE *FATB* GENE IN ARABIDOPSIS DEMONSTRATES AN ESSENTIAL ROLE OF SATURATED FATTY ACIDS IN PLANT GROWTH

ABSTRACT

Acyl-ACP thioesterases determine the amount and type of fatty acids that are exported from the plastids. In order to better understand the role of the FATB class of acyl-ACP thioesterases we identified an Arabidopsis mutant with a T-DNA insertion in the *FATB* gene. Palmitate (16:0) content of glycerolipids of the mutant was reduced 42% in leaves, 56% in flowers, 48% in roots and 56% in seeds. In addition, stereate (18:0) was reduced by 50% in leaves and 30% in seeds. The growth rate was reduced in the mutant resulting in 50 % less fresh weight at 4 weeks compared to wild-type plants. Furthermore, mutant plants produced seeds with low viability and altered morphology. Analysis of individual glycerolipids revealed that the fatty acid composition of prokaryotic plastid lipids was largely unaltered whereas the impact on eukaryotic lipids varied but was particularly severe for phosphatidylcholine (PC) with more than a 4-fold reduction of 16:0 and 10-fold of 18:0 levels. The total wax load of *fatb-ko* plants was reduced 20 % in leaves and 50% in stems, implicating FATB in the supply of saturated fatty acids for wax biosynthesis. Analysis of C_{18} sphingoid bases derived from 16:0 indicated that, despite a 50 % reduction in exported 16:0, the mutant cells maintained wild-type levels of sphingoid bases presumably at the expense of other cell components. Cutin composition of the mutant was reduced by more than 85 % in C_{16} derivatives, however its total cutin load was unaltered compared to wild type. The growth retardation caused by the *fatb*

mutation is enhanced in a *fatb-ko act1* double mutant where saturated fatty acid content is further reduced. Together, the results demonstrate the *in vivo* role of FATB as a major determinant of saturated fatty acid synthesis and the essential role of saturates for the biosynthesis and/or regulation of cellular components critical for plant growth and seed development.

INTRODUCTION

In plants, *de novo* fatty acid synthesis in plastids can be terminated either by the action of plastidial acyltransferases that transfer the acyl group of acyl-ACP to produce glycerolipids within the plastid (prokaryotic pathway) or by acyl-ACP thioesterases (FAT) that release free fatty acids and ACP. After export from the plastid, free fatty acids are re-esterified to coenzyme-A (CoA) to form the cytosolic acyl-CoA pool, which is primarily used for glycerolipid biosynthesis at the endoplasmic reticulum (ER) (eukaryotic pathway) (Figure 4) (Browse and Somerville, 1991). In Arabidopsis leaves, 18:1 and 16:0 are the major products of plastid fatty acid synthesis and approximately 60 % of these products are exported to the cytosol as free fatty acids. In other tissues or plant species, flux through the acyl-ACP thioesterase to the eukaryotic pathway is more predominant with 90 % or greater contribution. Therefore, thioesterases play an essential role in the partitioning of *de novo* synthesized fatty acids between the prokaryotic and eukaryotic pathways. Moreover, thioesterase substrate specificity determines the chain length and saturation level of fatty acids exported from the plastid (Pollard *et al.*, 1991). Based on

Figure 18 Schematic representation of the enzymatic reaction catalyzed by acyl-ACP thioesteases (FAT).

Acyl-ACP thioesterases or FAT enzymes (fatty acid thioesterase) hydrolyze the thioester bond between acyl carrier proteins (ACP) and fatty acids to release free ACP and free fatty acids inside the plastid. Free fatty acids are then exported as acyl-CoA molecules. In most plants the are two classes of FAT enzymes, A and B. The FATA class has higher *in vitro* specificity for unsaturated fatty acids (18:1) whereas FATB has more activity towards saturated fatty acids (14:0, 16:0 and 18:0). The X axis of the histograms represent the fatty acid attached to ACP to form the acyl-ACP substrate.

Acyl-ACP Thioesterases (FAT)
18:1-ACP
18:0-ACP
16:0-ACP
4:0-ACP
FAS
acyltransferases
F
A
T
18:1
18:0
16:0
18:1-CoA
18:0-CoA
16:0-CoA
Plastid
Cytosol
At-FATA (in vitro activity)
120
100
80
60
40
20
0
14:0 16:0 18:0 18:1
At-FATB (in vitro activity)
120
100
80
60
40
20
0
14:0 16:0 18:0 18:1

Figure 18

amino acid sequence comparisons and substrate specificity, two different classes of acyl-ACP thioesterases have been described in plants (Voelker *et al.*, 1997). The FATA class has highest *in vitro* activity for 18:1-ACP and much lower activity for saturated acyl-ACP substrates. Members of the second class of thioesterases, FATB, prefer saturated acyl groups but also have activity for unsaturated acyl-ACPs (Figure 18) (Doermann *et al.*, 1995; Voelker *et al.*, 1997; Salas and Ohlrogge, 2002).

In the Arabidopsis genome, there are two genes for *FATA* and a single gene for *FATB* (Beisson F., http://plantbiology.msu.edu/gene_survey/front_page.htm). All other higher plants that have been examined appear to express both classes of thioesterase (Mekhedov *et al.*, 2000). One salient question is why plants require two classes of acyl-ACP thioesterase and what individual role does each provide. The major exported fatty acids in Arabidopsis is 18:1 and based on *in vitro* activity, it can be predicted that FATA determines the *in vivo* levels of 18:1 that move out from the plastid (Salas and Ohlrogge, 2002). In the case of FATB, a previous antisense and over-expression study in Arabidopsis demonstrated that this enzyme is involved, at least in part, in the *in vivo* production of saturates in flowers and seeds (Doermann *et al.*, 2000). Similarly, downregulation of *FATB* expression in soybean also demonstrates partial reduction of seed palmitic acid (Buhr *et al.*, 2002; Wilson *et al.*, 2001) However, the origin of palmitic acid which remains following gene silencing procedures and the extent to which each class of thioesterase contributes *in vivo* to the production of exportable fatty acids by different tissues remains unresolved.

One possible role for two thioesterases is to provide control over the saturated/unsaturated balance of membrane fatty acids. The composition of almost all plant, animal and microbial membranes contain a mixture of saturated and unsaturated fatty acids. Such a mixture is believed essential to provide a balance of physical properties (e.g. fluidity), as well as a method to adapt to changes in environment (e.g. temperature) and to prevent phase transitions or lateral phase separations which are promoted by lipids with uniform fatty acid composition. However, as demonstrated by extensive feeding studies with microbial cells that depend on exogenous fatty acids for membrane synthesis (Walenga and Lands, 1975; Mc Elhaney, 1989) most organisms can accommodate a surprising range of fatty acid structures in their membranes without impairments in growth. Similarly, a wide range of mutations in plant fatty acid desaturases demonstrate that fatty acid composition of plant membranes can be considerably altered with no apparent phenotype under normal growth conditions. For example, Arabidopsis mutants with elimination of 16:1 *trans* Δ3, 16:3, 18:3, or large reductions in 18:2 grow normally at 25°C (Wallis and Browse, 2002). These studies suggest that within a certain range, the composition of fatty acids in membranes is not critical as long as a mixture of acyl chains is provided. However, beyond this range, e.g. under temperature extremes or when major changes in fatty acid composition occur, growth can be severely impacted. For example, the *fab2* mutant of Arabidopsis is severely reduced in growth due to an increase in18:0 leaf content to 10-15 % (Lightner *et al.*, 1994), and in the *fad2 fad6* double mutant, the complete elimination of polyunsaturated fatty acids leads to loss of photosynthetic ability (Wallis and Browse, 2002). In addition to a role as bulk components of membranes, where exact structures

seem less critical, some unsaturated fatty acids have more specialized roles as precursors for signal molecules (e.g. linolenic for jasmonate) and saturated fatty acids are precursors for sphingolipids, surface waxes and cutin, and are involved in protein acylation. Such roles are clearly vital because mutants completely lacking trienoic acids, for example, are male sterile and impaired in pathogen defense (Wallis and Browse 2002). To date such critical roles for saturated fatty acids have not been described.

In this study we describe the isolation and analysis of a mutant disrupted in the *FATB* thioesterase gene. Surprisingly, despite only an overall 40-50 % reduction of saturated fatty acid content, the mutant grew slowly and produced deformed seeds with low viability. These results have revealed an essential role of FATB in the supply of saturated fatty acids for the biosynthesis and/or regulation of components vital for plant growth and seed development.

RESULTS

Mutant Isolation and Complementation Analysis

In an effort to better understand the *in vivo* functions of the Arabidopsis *FATB* acyl-ACP thioesterase, a PCR-based strategy was used to screen a population of T-DNA tagged Arabidopsis plants (Sussman *et al.*, 2000) for disruption of the *FATB* gene. PCR analysis of pooled leaf genomic DNA identified a template from plants containing a T-DNA insertion in the second intron of the *FATB* gene (*fatb-ko*) (Figure 19). Genetic segregation analysis of heterozygote *FATB* T-DNA insertion lines selected one line segregating with

the expected ratio (3:1) for a single T-DNA insertion (280:105 BASTA resistant:susceptible) (χ^2 = 0.53, P > 0.4). However, since approximately half of the homozygous *fatb-ko* plants were lost during germination (see below) an expected ratio of 2.5:1 (resistant:susceptible) better fit the observed ratio (χ^2 = 0.18, P > 0.6). One hundred and ten individuals of these 280 resistant plants were grown to full maturity and of these, 25 had a slow-growth phenotype (see below). Again, the observed phenotypic segregation ratio agreed with the expected 2:0.5 ratio (χ^2 = 0.2, P > 0.6) of segregation for a single T-DNA insertion when considering the lower germination rate of the mutant. A subset of the 110 BASTA resistant plants were randomly selected and subjected to PCR and GC analysis to determine the genotype and the fatty acid composition, respectively. All plants with wild-type visual phenotype and fatty acid composition were heterozygous for the *FATB* T-DNA insertion, whereas plants with mutant visual phenotype and fatty acid composition were homozygous for the same insertion (data not shown). The confirmation that the visual phenotype and the genetic (T-DNA) and the biochemical (fatty acid composition) markers all co-segregated suggested that the T-DNA insertion in the *FATB* gene was responsible for both the observed growth and fatty acid phenotypes. In order to rule out the possibility that a second-site mutation closely linked to the *FATB* T-DNA insertion was responsible for the phenotype, the wild-type FATB cDNA was inserted under the control of the constitutive CaMV35S promoter and expressed in homozygous *fatb-ko* plants. Transgenic lines resistant to both hygromycin-B (transgene T-DNA) and BASTA (knockout T-DNA) were indistinguishable from wild-type and showed normal growth and biochemical characteristics (see below). Therefore,

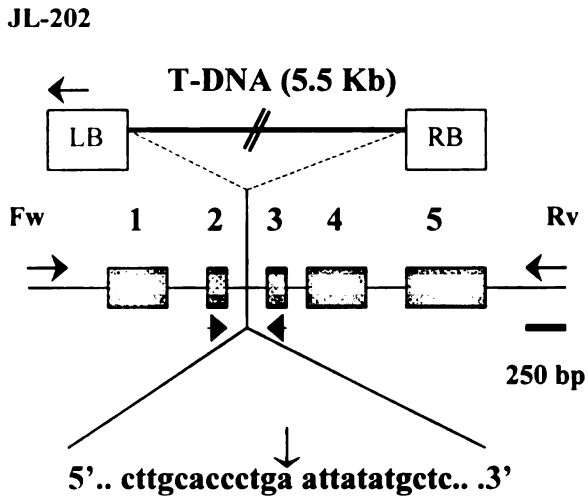

Figure 19. Structure of the Arabidopsis *FATB* gene carrying the T-DNA insertion.

A, The *FATB* gene is composed of 5 exons and the T-DNA is located in the second intron. The arrows above the gene scheme represent the primers used for the initial screening of the *fatb*-knockout plants (Fw: forward, Rv: reverse, JL-202: left border of T-DNA). The arrowheads underneath exons 2 and 3 represent the primers used for mRNA quantification by real-time PCR. B, Sequence of a portion of intron 2 encompassing the site of integration of the T-DNA (arrow)

we conclude that disruption of the *FATB* gene is responsible for the phenotypes observed in the mutant plants.

FATB mRNA Expression Analysis

The T-DNA was located in the second intron of the *FATB* gene and therefore it was possible that the cells could correctly splice out at least a fraction of the precursor FATB RNA to yield mature mRNA. Indeed, by using RT-PCR with a set of primers that spanned the second intron of the *FATB* gene (see Figure 19), small amounts of correctly

spliced mRNA were detected (data not shown). To determine the extent of gene disruption, the FATB transcript levels were quantified by real-time PCR in wild-type and mutant leaf tissue and found to be more than 150-fold lower in mutant than in wild-type (Table 6). Therefore, although PCR could detect correctly spliced FATB mRNA, this transcript represented less than 0.7 % of wild-type levels. Furthermore, western blot analysis developed with anti-Arabidopsis FATB antibodies did not detect FATB protein in the insertion mutant (data not shown). These results indicated that the T-DNA insertion generated an essentially complete knock-out mutant.

Table 6 Relative amounts of FATB mRNA in leaf tissue of wild-type Arabidopsis and *fatb-ko* mutant

	FATB mRNA		EIF4A1* mRNA		ratio FATB/eIF4A1
	C_t value	2^{Ct}	C_t value	2^{Ct}	
WT (Ws)	23.5 ± 0.4	$1.2\ 10^7$	21.4 ± 0.36	$2.8\ 10^6$	0.24
fatb-ko (het)	24.3 ± 0.1	$1.0\ 10^7$	21.2 ± 0.15	$2.3\ 10^6$	0.23
fatb-ko (hom)	30.4 ± 0.3	$1.4\ 10^9$	21.2 ± 0.18	$2.4\ 10^6$	0.0016

*The eukaryotic protein synthesis initiation factor A1 (eIF4A1) mRNA was used as an internal control since the levels of this transcript did not differ in leaf tissue of wild-type and *fatb-ko* plants. Het and Hom: heterozygous and homozygous for T-DNA insertion respectively.

FATB is Essential for Normal Seedling Growth

The first visual characteristic of *fatb*-knockout plants was their size compared to wild-type (Figure 20A and B). The rosettes of *fatb-ko* plants were approximately half the diameter of wild-type rosettes during the first weeks of growth at 22°C. In addition, the bolting time was delayed in the mutant. More than 90% of the wild-type plants bolted after 4 weeks under our growing conditions, whereas development was delayed in *fatb-ko* such that only after the sixth week did more than 90% of *fatb-ko* plants bolt (Table 7).

The morphology of the different organs from mutant plants was unchanged compared to wild-type. However the stems of the mutant elongated more slowly than wild-type stems. As shown in Figure 21A and B, a plot of the fresh weight of the aerial parts of wild-type and *fatb-ko* mutant plants indicated that during the first 4 weeks after germination the plants grew at a constant rate. However, the rate was slower for the mutant. Results in Figure 21B (log scale) indicated that wild-type plants increased their fresh weight 10.6-fold (± 0.4) per week, whereas for the *fatb-ko* plants the increase was only 8.8-fold (± 0.6). This 17 % reduction in growth rate led to a reduction of more than 50 % in the fresh weight of the mutant after 4 weeks (Figure 20A). Growth of both wild-type and mutant plants slowed after the fourth week, but more so for the former, such that by the sixth week the *fatb*-knockout plants differed in size and fresh weight by about 25% (Figure 21A). During the growing phase the percent ratio of dry to fresh weight remained at ~9 % while during the drying period it increased to ~14 % for both wild-type and mutant plants. Taken together, the similar morphology but different growth rates suggested that differences in wild-type and mutant plants were the consequence of a reduced growth rate and not altered development of the mutant. Wild-type and *fatb-ko* plants were grown in the presence of 1 % sucrose on either plates or liquid culture to determine if the normal growth rate could be recovered. Sucrose availability did not eliminate the slower growth rate of the mutant (data not shown) suggesting that photosynthetic capacity or carbon limitations of the *fatb*-knockout plants were not causes of the reduced growth rate.

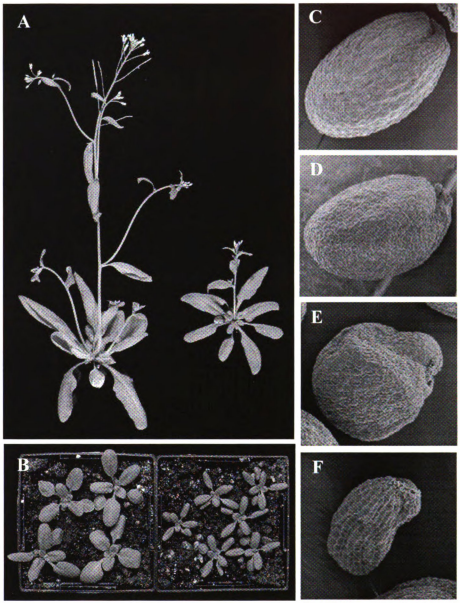

Figure 20. Growth and morphology of Arabidopsis wild-type and *fatb-ko* plants and seeds. A, Four weeks old wild-type (left) and *fatb-ko* (right) plants. B, Two weeks old wild-type (left) and *fatb-ko* (right) plants. C, wild-type Arabidopsis seed. D, E and F, wild-type-like, intermediate deformed and very deformed seeds from *fatb-ko* plants respectively.

Plant growth rate is modified by temperature and part of this effect may be associated with variations in the physical properties of cell membranes. To test the possibility that the growth retardation of the *fatb-ko* mutant was a function of temperature, wild-type and *fatb-ko* plants were grown for 2 weeks at 22°C and then transferred to three different temperatures (16°C, 22°C and 36°C). The *fatb-ko* plants showed the same percent reduction (~50 %) of fresh weight per seedling compared to wild-type at the three different temperatures. Therefore, the slower relative growth of the mutant plants was not altered within the range of temperatures used. In addition we tested wether adding exogenous saturated fatty acids by either spraying plants or supplementing seedlings grown in liquid culture could overcome the slower growth of the *fatb-ko* mutant. These procedures were not sufficient to chemically complement the *fatb-ko* phenotype. In fact, addition of higher amounts of exogenous fatty acids showed deleterious effects on plant growth (data not shown).

Leaf and chloroplast morphology of wild type Arabidopsis and *fab-ko*

The leaves of *fatb-ko* average 50 % of the area and fresh weight of wild type leaves (Figure 20 and data not shown). These differences may be brought about by reduced cell size or less number of cells per leaf. In addition, leaves are composed of different cell types (e.g., mesophyll, epidermal) and some of those might be differentially affected by the genetic lesion. We analyzed optical sections of leaf tissue from wild type and mutant plants by laser confocal microscopy (LCM) and leaf epidermal cells by scanning electron microscopy (SEM) (Figure 22A to D). The structures of leaves and epidermis together

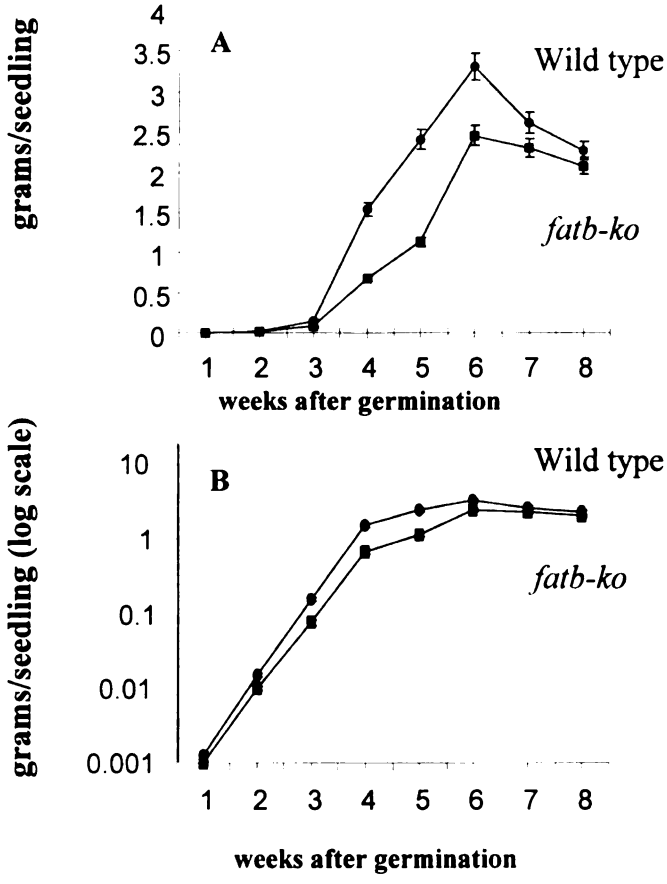

Figure 21. Growth curves of Arabidopsis wild-type and *fatb-ko* plants.
A, The fresh weight of aerial parts of Arabidopsis plants was measured on a weekly basis for a period of 8 weeks. Each time point is the average value of at least 7 individual plants. B, Logarithm of the fresh weight versus time.

with sizes of epidermal and mesophyll (palisade and spongy) cells were similar between both plant classes (Figure 22A to D). Thus, these observations indicated that the smaller size of *fatb-ko* leaves compared to wild type was the result of fewer cells per leaf rather than smaller cell sizes. These results contrasted with those obtained with the Arabidopsis *fab2* mutant, in which decrease in leaf size was the consequence of reduced

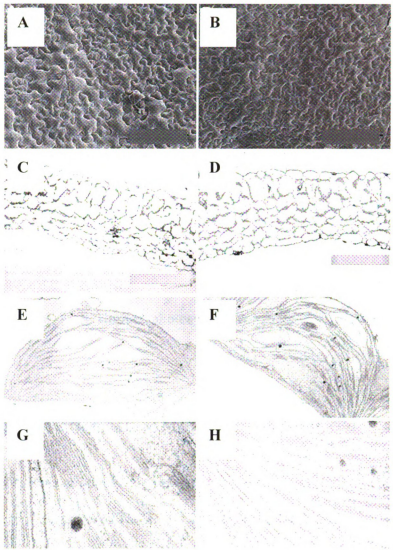

Figure 22. Microscopic analysis of leaf section and chloroplast structure of wild type and *fatb-ko* seedlings. A-B, Scanning electron micrographs of wild type (A) and *fatb-ko* (B) leaf epidermal cells. C-D, leaf sections of wild type (C) and *fatb-ko* (D). E-F, Transmission electron microscopy (TEM) of wild type (E) and *fatb-ko* (F) leaf chloroplasts. G-H, TEM of thylakoid membranes from wild type (G) and *fatb-ko* (H) leaf chloroplasts.

expansion of leaf cells (Lightner et al., 1994).

Growth of *fatb-ko* in the presence of sucrose did not revert its growth phenotype, suggesting that photosynthetic capabilities of the mutant did not differ substantially from wild type plants. Analysis of leaf chloroplast ultrastructure by transmission electron microscopy (TEM) showed no obvious differences between organelles of wild type and *fatb-ko* (Figure 22E and F). The size and number of chloroplasts per cell of the mutant were similar to wild type. Moreover, structure and number of thylakoid membranes were also similar between the two plant classes (Figure 22G and H). In summary, these observations agreed with similar photosynthetic capabilities between wild type and mutant plants.

FATB is Essential for Normal Seed Morphology and Germination

Germination of seeds produced by *fatb-ko* plants was reduced by approximately 50 % either on soil or 1 % sucrose (Table 7). Close examination of mature seeds produced by the *fatb-ko* mutant revealed a continuous range of deformity in seed morphology, with wild-type-like seeds in one extreme to very deformed seeds (approximate frequency of 20 %) in the other (Figure 20C-F and Table 7). The germination rate of very deformed seeds was only 16 %. These observations suggested that some stage of seed or embryo development may be substantially affected in the mutant. By analyzing developing siliques it was not evident that deformed seeds were located in specific segments of this organ. Upon surface sterilization, some mutant seeds also lost the seed coat, suggesting

Table 7. Arabidopsis wild-type and *fatb-ko* bolting time and germination rates.

Bolting time* (%)		
Time	WT (WS)	*fatb-ko*
4th week	92.3	27.6
≥ 5th week	7.7	72.4
Germination rate (%)		
	WT (WS)	*fatb-ko*
Soil	96.7	45.6
1% sucrose	94.4	45.8
Deformed seeds in total seeds (%)		
	WT (WS)	*fatb-ko*
	0.8	21.7
Germination on soil of deformed seeds (%)		
		fatb-ko
		16

* 18:6 h light:dark; 22 C

that the structure of this tissue could be altered. Scanning electron microscopy (SEM) analysis of the seed coat from mutant plants with wild-type-like or intermediate morphology did not show any obvious structural differences compared to wild-type seeds (Figure 20C-F). Many deformed seeds from the mutant displayed a shriveled seed coat (Figure 20F).

Fatty Acid Composition of *fatb-ko* Tissues.

As indicated in Table 8, palmitate (16:0) in homozygous *fatb-ko* plants was reduced 42 % in leaves, 56% in flowers, 48% in roots and 56% in seeds compared to wild-type. Stearate (18:0) decreased almost 50 % in leaves and 30 % in seeds, with negligible changes in flowers and roots. The *fatb-ko* plants also showed an increase of 150-200 % in oleate (18:1) and 40-60 % in linoleate (18:2) in leaves, flowers and roots. Linolenate (18:3) declined by 15-20 % in leaves, flowers and roots. Seed unsaturated fatty acids were less affected. Together, these results demonstrate the *in vivo* role of FATB as a major determinant of 16:0 in all the tissues analyzed and also indicate that FATB contributes to the level of 18:0 in leaves and seeds. *fatb-ko* plants transformed with the wild-type FATB cDNA under the CaMV35S constitutive promoter had a fatty acid composition very similar to wild type, confirming that the FATB cDNA complemented the biochemical phenotype of the mutant (Table 8).

Table 8. Fatty acid composition of wild-type and *fatb-ko* Arabidopsis tissues (mol%).

	16:0	16:1(9)	16:1(3)	16:3	18:0	18:1(9)	18:2	18:3	Other
Leaf tissue									
WT (Ws)	17.5	0.6	3.7	11.8	1.5	2.6	14.4	47.7	
fatb-ko	10.1	1.3	3.4	12.4	0.8	8.2	23.2	40.1	
35S-FATB**	15.5	0.6	4.0	11.9	1.3	4.6	16.8	45.0	
Flowers									
WT (Ws)	27.4	1.7		1.2	2.4	3.2	30.6	31.3	
fatb-ko	12.0	4.6		2.6	2.6	8.5	41.9	24.7	
Roots									
WT (Ws)	25.3	0.9			6.6	3.6	28.8	21.3	13.2
fatb-ko	13.4	1.1			6.0	9.2	42.5	17.4	10.1
Seeds									
WT (Ws)	8.3	0.3			3.5	13.0	27.5	18.6	28.6
fatb-ko	3.6	0.4			2.4	11.9	33.1	16.2	32.1

* Average of at least 3 samples. Standard deviation values are not shown and represent less than 5% of the average values. ** *fatb-ko* plants transformed with CaMV35S-FATB cDNA.

Fatty Acid Composition of Individual Leaf Glycerolipids.

The fatty acid composition of individual glycerolipids from homozygous *fatb-ko* and wild-type leaves are presented in Table 9. Palmitate reductions occurred mainly in extra-plastid lipids. While phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinisitol (PI) had an approximately 50 % reduction in 16:0 compared to wild type, in phosphatidylcholine (PC) the reduction was almost 80 %. The palmitate levels in plastid lipids were less affected with a significant reduction (40 %) only in sulfoquinovosyldiacylglycerol (SQDG). All of the extra-plastid glycerolipids except PI had reduced 18:0. Again PC was the most affected with a 10-fold reduction in 18:0. The characteristic changes in unsaturated fatty acids seen in Table 8 for total leaf lipids, namely increases in 18:1 and 18:2, and a decrease in 18:3, were most pronounced for the phospholipids and SQDG. The data in Table 9 also indicated that despite the changes in fatty acid composition there were no major differences in the relative proportions of leaf glycerolipids between wild-type and *fatb-ko* plants. And finally, the total amount of fatty acid methyl esters produced by acid-catalyzed transmethylation of Arabidopsis leaves was the same for the wild-type (11.5 μmoles/gfw) and *fatb-ko* (11.6 μmoles/gfw), indicating that the *fatb-ko* did not affect net fatty acid accumulation per fresh weight.

Acyl-ACP Thioesterase Activity

To determine if any compensatory changes occurred in acyl-ACP thioesterase activity, mutant and wild-type plants were assayed for hydrolysis of 18:1-ACP and 16:0-ACP. The *FATA* gene product has an acyl specificity 18:1 >> 18:0 >> 16:0 while the *FATB*

Table 9. Fatty acid composition of leaf glycerolipids of Arabidopsis wild-type (WS) and *fatb-ko* mutant (mol%).

	% total	16:0	16:1(3)	16:3	18:0	18:1(9)	18:1(11)	18:2	18:3
PC									
WT (WS)	11.2 ± 0.3	21.1			2.4	6.1	-	34.7	35.5
fatb-ko	13.6 ± 0.7	4.5			0.2	17.8	1.3	46.1	30.1
PE									
WT (WS)	10.0 ± 0.2	29.0			2.1	2.8	-	35.1	30.8
fatb-ko	7.9 ± 0.6	11.6			1.1	11.3	1.2	50.8	24.9
PS									
WT (WS)	0.9 ± 0.1	30.8			6.8	2.9		27.1	32.3
fatb-ko	0.8 ± 0.1	17.2			2.9	10.2		44.3	25.2
PI									
WT (WS)	5.5 ± 0.1	40.0			2.0	2.2		25.3	30.3
fatb-ko	4.4 ± 0.1	21.2			1.8	9.2		36.9	29.3
PG									
WT (WS)	12.0 ± 0.3	29.2	22.0		1.1	4.5	-	10.8	32.1
fatb-ko	12.6 ± 0.3	24.3	20.7		-	8.5	0.9	16.9	27.6
SQDG									
WT (WS)	4.3 ± 0.1	34.6			1.5	3.6	0.7	21.5	37.8
fatb-ko	4.2 ± 0.6	20.4			0.5	10.5	1.2	36.4	30.8
DGDG									
WT (WS)	20.2 ± 0.3	12.8		4.2	1.0	1.1	-	6.7	73.9
fatb-ko	19.4 ± 0.1	12.0		5.4	0.5	1.2	0.5	7.9	70.5
MGDG									
WT (WS)	35.5 ± 0.1	1.6		38.1		0.8		3.0	53.4
fatb-ko	36.7 ± 0.2	1.3		40.3		1.6		3.2	50.7

gene product has a specificity 16:0 > 18:1 > 18:0 (Doermann *et al.*, 1995; Salas and Ohlrogge, 2002). The activity from the *FATA* gene product dominates acyl-ACP thioesterase activity measurements made with crude extracts. Oleoyl-ACP hydrolytic

activity in leaf extracts of wild-type and mutant plants was similar (~125 pmol*min^{-1}*mg^{-1}) and hydrolytic activity on 16:0-ACP was close to background levels and therefore difficult to quantify. These results indicate that measurable acyl-ACP hydrolytic activity does not change in mutant leaf extracts compared to wild-type and therefore endogenous levels of FATA activity are not up-regulated in the mutant.

Total Palmitate Content in Arabidopsis Leaf Tissue

Most acid or base-catalyzed transmethylation methods for fatty acid analysis efficiently convert O-acyl groups such as found in glycerolipids to fatty acid methyl esters. However, N-acyl groups such as found in sphingolipids react only very slowly using such methods. In order to evaluate the total palmitic and stearic content (O- and N-linked) of Arabidopsis cells, a strong alkaline hydrolysis on total leaf tissue, on lipids extracted with multiple chloroform methanol extractions, and on the solvent-extracted residue was performed. The total 16:0 content in leaves of wild-type plants was 1.87 ± 0.02 μmoles/gfw, whereas leaves of *fatb-ko* plants contained 1.14 ± 0.03 μmoles/gfw. Thus, a 39 % reduction in total 16:0 was observed in the mutant, similar to the 42 % reduction of 16:0 in glycerolipids (Table 8). The amount of total stearic acid was also reduced by 50 % in leaf tissue, from 0.16 ± 0.01 μmoles/gfw in wild-type plants to 0.075 ± 0.005 μmoles/gfw in the mutant. The analysis of extracted lipids and solvent-extracted residue indicated that almost all the 16:0 and 18:0 in the cells was co-extractable from leaf tissue, since the lipid fraction contained similar amounts of saturated fatty acids to the total tissue (data not shown). In contrast, the solvent-extracted residue which may contain acylated proteins and other insoluble lipids, contained 3 % of the total 16:0 and no

detectable 18:0. Similar reductions of 16:0 and 18:0 were observed in all the fractions analyzed, indicating that absence of FATB reduced saturated fatty acid levels in both organic soluble and insoluble components.

Leaf and Stem Surface Wax Analysis

The very long chain fatty acids (VLCFA) required for wax synthesis are produced by elongation of 16 and 18 carbon saturated fatty acids (Post-Beittenmiller, 1996). To determine if the 40-50 % reduction in saturated fatty acids influences total wax load or composition in the *fatb-ko* mutant, leaf and stem epicuticular waxes were analyzed. The results in Table 10 indicated that at 5 weeks total wax load per fresh weight in leaves was reduced by 20 % in the *fatb-ko* mutant. However, no novel components or substantial changes in the distribution of wax components were observed at this stage. A 20 % reduction in leaf wax load was consistently observed at different stages of plant development (data not shown). Analysis of primary stems indicated a 50 % reduction in wax load per fresh weight in the *fatb-ko* mutant compared to wild type (1.1 ± 0.1 μmol/gfw versus 2.2 ± 0.3 μmol/gfw respectively), again without changes in the distribution of wax components. These data indicate that supply of saturated fatty acids by FATB is one factor limiting wax biosynthesis but that reduction of this supply does not result in the replacement of 16:0 by 18:1 or other precursors for surface wax structures. The role of FATB in supplying wax precursors was more evident in stems than in leaves as the former tissue accumulates higher amounts of epicuticular waxes. Similar tissue specific wax reductions are observed in most of the Arabidopsis wax biosynthetic mutants (*eceriferum*) in which reductions in stem waxes are larger than in

Table 10. Major components of epicuticular leaf waxes from wild-type Arabidopsis and *fatb-ko* mutant (mol%).

(carbon chain length)	WT (WS)	*fatb-ko*
Alkanes		
27	1.4 ± 0.1	1.2 ± 0.05
29	21.9 ± 1.2	22.1 ± 0.1
30	1.2 ± 0.1	1.0 ± 0.03
31	39.4 ± 1.1	40.5 ± 0.5
32	0.9 ± 0.1	0.9 ± 0.03
33	11.7 ± 0.4	12.7 ± 0.03
Free fatty acids		
24	3.5 ± 0.2	3.3 ± 0.06
26	5.7 ± 0.2	5.3 ± 0.09
28	2.2 ± 0.4	1.8 ± 0.1
30	0.7 ± 0.1	0.5 ± 0.1
Primary alcohols		
26	3.1 ± 0.4	2.3 ± 0.3
28	3.6 ± 0.5	3.6 ± 0.3
30	2.2 ± 0.1	2.2 ± 0.02
32	2.3 ± 0.1	2.2 ± 0.02
Total (μg/gfw)	104.1 ± 6.6	83.9 ± 4.5
Total (μmol/gfw)	0.24 ± 0.01	0.19 ± 0.01

leaf waxes (Rashotte *et al.*, 2001).

Sphingoid Base Analysis

Since sphingoid base and sphingolipid synthesis are initiated by serine palmitoyltransferase (Lynch, 1993) an analysis of sphingoid bases was conducted. Leaves of wild-type (0.54 ± 0.09 μmoles/gfw) and *fatb-ko* (0.50 ± 0.08 μmoles/gfw) plants did not differ significantly in the total amount of sphingoid bases. However,

differences could be observed in the relative abundance of the individual sphingoid bases (Table 11). Most significantly, trihydroxy-18:0 (t18:0) increased by almost 4-fold in the total sphingoid bases of the *fatb-ko* mutant. The total sphingoid base composition was similar to that reported by Sperling *et al.* (1998): the most abundant sphingoid base in Arabidopsis leaf tissue was t18:1(8E), followed by t18:1(8Z). Monoglucosylceramide (MGC) is considered one of the most abundant sphingolipids and therefore the sphingoid base composition in leaf MGC was analyzed. The base composition in MGC of wild-type and mutant plants was indistinguishable, and was similar to that reported by Imai *et al* (2000) (Table 11). The difference in composition between total and MGC sphingoid base compositions indicates that MGC is not the predominant sphingolipid in Arabidopsis leaves. As suggested by Imai *et al.* (2000), complex sphingolipids such as phosphoinositolceramides could be more abundant than MGC in Arabidopsis leaf tissue. Sphingoid bases in extracted lipids and solvent-extracted residue were also analyzed. The latter fraction can contain sphingoid bases from highly glycosylated phophoinositolceramides and GPI moieties from GPI-anchored proteins. Again, no major changes in sphingoid base composition between wild-type and *fatb-ko* plants were observed except in trihydroxy 18:0 (Table 11).

Leaf and stem cutin analysis

The monomers of cutin are synthesized from the CoA esters of palmitic acid (16:0) and oleic acid (18:1), respectively. To determine if the reduction in saturated fatty acids in *fatb-ko* influences cutin composition, leaf and stem cutin were analyzed. The results shown in Figure 23 indicate that 16 carbon derivatives of cutin were reduced by more

Table 11. Sphingoid base content of leaf tissue from wild-type Arabidopsis and *fatb-ko* plants (mol%)

	t18:1(8E)	t18:1(8Z)	t18:0	d18:1(8E)	d18:1(8Z)	d18:2(4,8)
Total Tissue						
WT (WS)	60	32	2	4	t	2
fatb-ko	57	32	8	2	1	1
Monoglucosylceramide						
WT (WS)	24	59	t	11	t	5
fatb-ko	25	59	t	10	2	5
Solvent Extracted Lipids						
WT (WS)	62	33	2	2	1	t
fatb-ko	57	36	5	2	t	t
Solvent-Extracted Residue						
WT (WS)	85	7	3	t	3	1
fatb-ko	76	7	13	t	3	1

Dihydroxy bases: 8-sphingenine (d18:1[8E or Z]), 4,8-sphingadienine (d18:2[4E,EZ or 4E,8Z]); trihydroxy bases: 4-hydroxysphinganine (t18:0) and 4-hydroxy-8-sphingenine (t18:1[8E or Z]). t, traces.

than 85 % in fatb-ko leaves and stems compared to wild type. These results demonstrated that FATB provides 16:0 for cutin biosynthesis in Arabidopsis. Although fatb-ko has reduced 16 carbon derivatives in cutin the total cutin load was unaffected in the mutant (approximately 5 umol/g residue in leaves and stems of both plant classes). Thus, the mutant compensates for the decrease in C_{16} derivatives by producing more C_{18} derivatives (Figure 23).

fatb-ko act1 double mutant

The lipid analysis demonstrated that despite the absence of FATB in the plastids, mutant plants contain approximately 50 % of the saturated fatty acids found in wild-type plants. What is the origin of these remaining saturated fatty acids? Palmitate and stereate, as 16:0-ACP and 18:0-ACP respectively, may be utilized directly by acyltransferases in the plastid for prokaryotic lipid synthesis (Somerville *et al.*, 2000). In order to evaluate how much of the saturated fatty acids remaining in the *fatb-ko* plants derive from acyl group fluxes through plastidial acyltransferases, a cross between *fatb-ko* plants and the Arabidopsis *act1 (ats1)* mutant was performed. The *act1* mutant has reduced plastidial glycerol-3-phosphate:acyl-ACP acyltransferase activity, the first step in the plastid pathway of glycerolipid biosynthesis. Although *act1* plants contain reduced amounts of 16:3 and higher amounts of 18:1, growth of this mutant is normal (Kunst *et al.*, 1988). However, the *fatb-ko act1* double mutant was severely impaired in growth when compared to wild-type, *fatb-ko* and *act1* plants (Figure 24). Leaf fatty acid analysis of the double mutant indicated that this tissue contained approximately 3-4 mol% 16:0, representing a 70 % reduction compared to wild type (Table 8 and 12). The levels of 18:1 in leaves were increased in the double mutant compared to *fatb-ko* whereas 18:2 and 18:3 levels remained almost unchanged (Table 8 and 12). Interestingly, leaf levels of 18:0 in the double mutant were similar to those in *fatb-ko* leaves, indicating no further decrease in overall 18:0 by the second mutation. Analysis of individual lipid classes from leaf tissue of the double mutant revealed that the C_{16} acyl composition of extraplastidial glycerolipids and PG did not differ substantially from those in *fatb-ko* leaves (data not

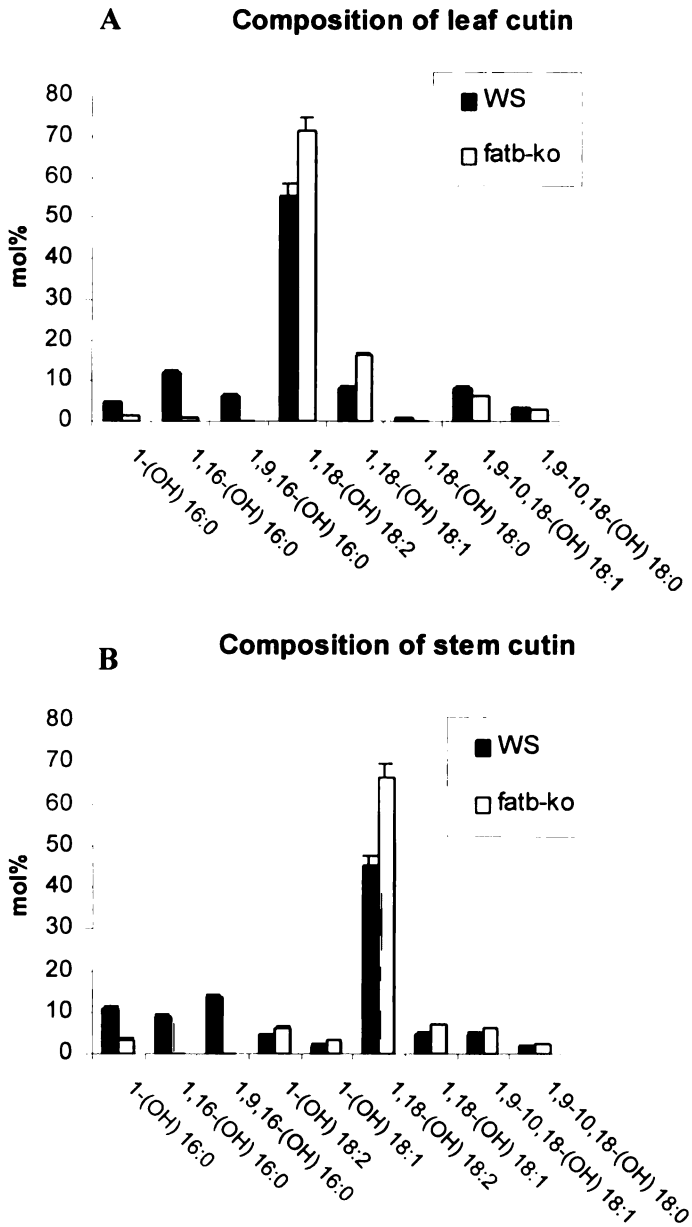

Figure 23. Leaf and stem cutin composition of wild type Arabidopsis and *fatb-ko* (mol%).

Cutin components were reduced with $LiAlH_4$ and analyzed by GLC. **A,** cutin monomer composition of four-week old leaves from wild type and fatb-ko plants. **B,** cutin monomer composition of six-week old stems from wild type and fatb-ko plants. Bars denote standard deviations.

shown). Moreover, the relative abundance of each glycerolipid was similar. These results demonstrated that the second mutation (*act1*) affected primarily 16:0 accumulation in plastidial glycerolipids with minor effects on extraplastidial lipids compared to *fatb-ko*.

In summary, double mutant plants blocked in both FATB and ACT1 were further reduced in saturated fatty acid content to levels ~ 30 % of wild-type plants. These plants displayed a smaller size and thus this more severe growth phenotype associated with a greater reduction in saturated fatty acids further demonstrates the essential role of saturated fatty acids in maintaining normal rates of plant growth.

DISCUSSION

Acyl-ACP thioesterases are responsible for the export from the plastid of fatty acids produced by the *de novo* fatty acid synthesis system. In this study an Arabidopsis insertion mutant of the *FATB* gene was isolated, and we describe its effects on plant growth and on the production and utilization of saturated fatty acids. In a previous study, antisense of Arabidopsis FATB using the CaMV35S promoter resulted in a substantial reduction of 16:0 only in flowers and seeds and minimal difference in leaves and roots and did not show any visual phenotype (Doermann *et al.*, 2000). The lack of anti-sense impact on leaves suggested either FATB was not the major controller of 16:0 in leaves or that the reduction of FATB mRNA was not sufficient to reduce 16:0 levels in certain tissues. The characterization of an Arabidopsis *fatb*-knockout mutant in this study clarifies that the second interpretation is correct and that FATB is a major control point

Figure 24. Growth and morphology of Arabidopsis wild-type, *fatb-ko*, *act1* and *fatb-ko act1* plants.

Four weeks old wild-type (WS) (bottom right), *fatb-ko* (bottom left), *act1* (top right) and *fatb-ko act1* double mutant (top left) plants. The size of *act1* mutants is similar to the size of wild type Columbia (Col) plants (not shown). The size of wild type plants derived from the cross between *fatb-ko* (WS) and *act1* (Col) is intermediate to the parental ecotypes (not shown).

for saturated fatty acid fluxes in all tissues. The more extensive biochemical phenotype and the reduced growth rate and seed viability observed in the mutant compared to antisense plants suggest that the FATB enzyme or its mRNA may be in large excess and

Table 12. Leaf fatty acid composition of wild type Arabidopsis (Columbia), *act1* and *fatb-ko act1* double mutant (mol%)

	16:0	16:1	16:1(3)	16:3	18:0	18:1(9)	18:2	18:3
Wild type (Col)	16.2	1.4	3.0	11.3	1.5	3.0	13.8	49.8
act1	14.3	1.1	3.1	0.4	1.6	11.9	24.1	43.1
fatb-ko act1	3.1	1.2	3.2	0.5	0.8	23.4	24.8	41.5

difficult to reduce sufficiently by antisense methods to produce a growth phenotype.

Seedling Growth and Seed Development

A large number of mutants with diverse changes in fatty acid composition have been isolated in Arabidopsis (Wallis and Browse, 2002). Most of these are not readily distinguishable from wild-type plants when grown at standard temperatures (15°C-25°C). One of the few examples in which a mutation affecting fatty acid composition has consequences for plant growth is the *fab2* mutant in Arabidopsis, in which elevated levels of 18:0 in the membrane lipids results in dwarf plants (Lightner *et al.*, 1994). However, the phenotype can be partially ameliorated by growing the mutant at high temperature, suggesting that membrane fluidity or a related physical property reduces growth of the mutant at 22°C.

The Arabidopsis *fatb*-knockout is the first example of an Arabidopsis mutant with reduced levels of saturated fatty acids where a reduction in vegetative growth at standard growth conditions occurred. Low temperature did not alleviate nor did high temperature

exacerbate the slow growth phenotype of *fatb-ko* plants suggesting that effects other than changes in bulk membrane physical properties were limiting the growth of the mutant. Hence, in contrast with *fab2* in which high 18:0 levels may disrupt the proper function of membranes, reduced saturate levels in *fatb-ko* plants may be altering the biosynthesis and function of critical cell components. However, we can not rule out that lower amounts of saturates may be associated with more subtle changes in the physical properties of cellular membranes that could affect functions such as transport or vesicle formation. The *fatb-ko* mutant with a ca. 50% reduction in palmitate also contrasts with the *fab1* mutant that is increased approximately 50% in palmitate but displays normal growth (Wu *et al.*, 1994). The *fatb-ko* mutant is also distinguished from other fatty acid mutants in its effect on seed development and germination (Figure 20C-F and Table 7). However, in contrast to the slow growth phenotype that occurred in all seedlings, the penetrance of the seed phenotype was incomplete. At this stage it is not clear whether the seed defects are a consequence of alterations during specific seed developmental phases, or perhaps an indirect effect due to deficiencies in supply of nutrients from maternal tissues.

Palmitoyl-ACP pools in the plastid are subject to three major reactions; acyltransfer to glycerol, elongation to 18:0-ACP or hydrolysis by FATB. Mutations that block or reduce all three of these fates are now available. In the *fab1* mutant (Wu *et al.*, 1994), reduction in 16:0-ACP elongation results in increased 16:0 in both the prokaryotic and eukaryotic lipids suggesting that flux into both these pathways can be increased by increased availability of 16:0-ACP. Moreover, in both *fatb-ko* and *fab1* leaves there is an increase in 16:1(9) levels, suggesting that 16:0-ACP pools are increased within chloroplasts of

these two mutants. In contrast, in the *act1* mutant the loss of the acyltransferase pathway results in increased 16:0-ACP elongation to 18:0 rather than increased flux to the eukaryotic path via the FATB thioesterase (Kunst *et al.*, 1989). Similarly, in the *fatb-ko*, the reduction in flux via the thioesterase also primarily increased elongation to C_{18} rather than increased flux of C_{16} into prokaryotic lipids. These contrasting responses suggest that the elongation rate of 16:0-ACP is regulated primarily by availability of substrate but that the contributions of the FATB and acyltransferase reactions to 16:0 flux likely have additional levels of control.

Reduced Export of Palmitate in *fatb-ko* plants and Other Sources of Palmitate and Stearate in the Cell

Lipid analysis (Table 8) demonstrated that despite the homozygous *fatb-ko* insertion, mutant plants still produce approximately 50 % of the palmitate found in wild-type plants. How and where is the remaining palmitate balance in a plant cell produced? A similar statement and question can be made for stearate. Palmitate is both an intermediate and an end product of *de novo* fatty acid synthesis in the plastid. Palmitate, as 16:0-ACP, may be utilized directly by acyltransferases in the plastid for prokaryotic lipid synthesis, in particular by the lyso-phosphatidic acid sn-2 acyltransferase (Frentzen *et al.*, 1983). Alternatively, after hydrolysis by acyl-ACP thioesterases, free fatty acids including palmitic acid may be exported from the plastid, a proposed mechanism now substantiated by *in vivo* labeling (Pollard and Ohlrogge, 1999). And finally, plant mitochondria have the capacity for *de novo* synthesis of fatty acids (Wada *et al.*, 1997) and, although considered a minor pathway this organelle could partially compensate for low 16:0 levels in the *fatb* mutant. Could (1) the transfer of palmitate from prokaryotic lipids to

eukaryotic lipids, (2) the FATA acyl-ACP thioesterase, and/or (3) mitochondrial fatty acid synthesis account for the remaining exported palmitate production?

The cross of *fatb-ko* with *act1* plants demonstrates that the prokaryotic pathway provides about 60 % of the saturated fatty acids in leaves of *fatb-ko*. Approximately half of the saturates that are still produced in the double mutant can be attributed to plastidial PG (produced with prokaryotic character by an unknown path) and to FATA activity. The Arabidopsis FATA-encoded thioesterase has a small but measurable *in vitro* activity towards 16:0 and 18:0-ACP (about 2 % and 16 % of the activity towards 18:1-ACP, respectively) and our results suggest that these enzymes have *in vivo* hydrolytic activity towards 16:0- and 18:0-ACP, a conclusion also drawn by Nadev *et al.* (1992). The mutants studied here demonstrate that FATA, mitochondrial FAS or other sources of 16:0 are minor contributors to leaf saturated fatty acid flux in comparison to FATB and the plastid acyltransferases.

As summarized in Figure 25, the results of this study allow a better estimate of the relative contributions of alternative pathways for saturated fatty acid supply in plants. In wild-type plants, the total C_{16} fatty acids incorporated into membrane glycerolipids is 33.3 units, which includes 16:1(3)-trans in PG and 16:2 and 16:3 in MGDG. About 23 units are used for prokaryotic lipid synthesis, assuming that all PG and MGDG are derived from this pathway, and that the proportion of 16:0-containing DGDG that is prokaryotic is one third, based on its *sn*-2 distribution (Kunst *et al.*, 1989). Assuming that half the SQDG is of prokaryotic origin, of the remaining 10.3 units which are exported

from the plastid, about 2.5 units return to this organelle as SQDG and DGDG while the remaining 7.8 units are used for phospholipid synthesis (Figure 25). In the *fatb-ko* line we do not know what proportion of the 16:0-containing DGDG and SQDG pools are now of prokaryotic origin, so estimates are ranges. About 2.6-4.2 units of palmitate (25-45 % of wild-type) are exported and 2.6 units (33 % of wild-type) are retained for phospholipid synthesis while 0-1.6 units (0-65 % of wild type) are returned for plastid lipid synthesis. Palmitate used for prokaryotic lipid synthesis is barely affected by the mutation, increasing from 23 to 23.6-25.6 units. In the *fatb-ko act1* double mutant, about 3.5 units of palmitate (35 % of wild type) are exported and 2.5 units (33 % of wild type) are retained for phospholipid synthesis while 0-1 units (0-40 % wild type) are returned to the plastid.

Partition of Palmitate and Stearate to Non-glycerolipid Products.

Palmitate constitutes the primary saturated fatty acid exported from plastids and incorporated into membrane glycerolipids. In addition to glycerolipids, several other cellular components derived from the exported palmitate and/or stearate have essential structural and perhaps signaling roles for cell growth. First, sphingoid bases in plants are 18 carbon amino alcohols that are synthesized outside the plastid from palmitoyl-CoA and serine by the action of serine palmitoyltransferases (Lynch, 1993). In addition, 16:0

Figure 25. Simplified scheme of predicted fluxes of C_{16} and C_{18} fatty acids in membrane leaf glycerolipids of Arabidopsis wild type, *fatb-ko* and *fatb-ko act1* mutants.

The numbers in the figure represent average values of mol% units of C_{16} and C_{18} fatty acids accumulated in membrane glycerolipids. A range of values is given when alternative biosynthetic pathways are considered (see text for details). The average mol% units were calculated based on the mol% abundance of a particular fatty acid in a specific lipid, the relative abundance of the lipid and the contribution of each pathway (prokaryotic and eukaryotic) to the synthesis of that lipid (Browse *et al.*, 1986) (see Discussion for more details). Arrows represent the flux of acyl molecules through FATB and FATA (acyl-ACP thioesterases B and A respectively), KAS-II (3-ketoacyl-ACP synthase-II) and LPAAT (lyso-phosphatidate sn-2 acyltransferase) that are either used for lipid biosynthesis in the plastid (prokaryotic pathway) or exported from the same organelle. ACT1 (glycerol-3-phosphate acyltransferase) transfers 18:1 to glycerol-3-P in the prokaryotic pathway. Question marks indicate that: 1, the actual flux of 16:0 through FATA in wild type leaves is not known and an upper limit of 4 mol% units can be estimated based on *fatb-ko* data (however, flux through FATA in *fatb-ko* could be a compensatory mechanism); and 2, alternative pathways for biosynthesis of PG in the *fatb-ko act1* double mutant can be considered.

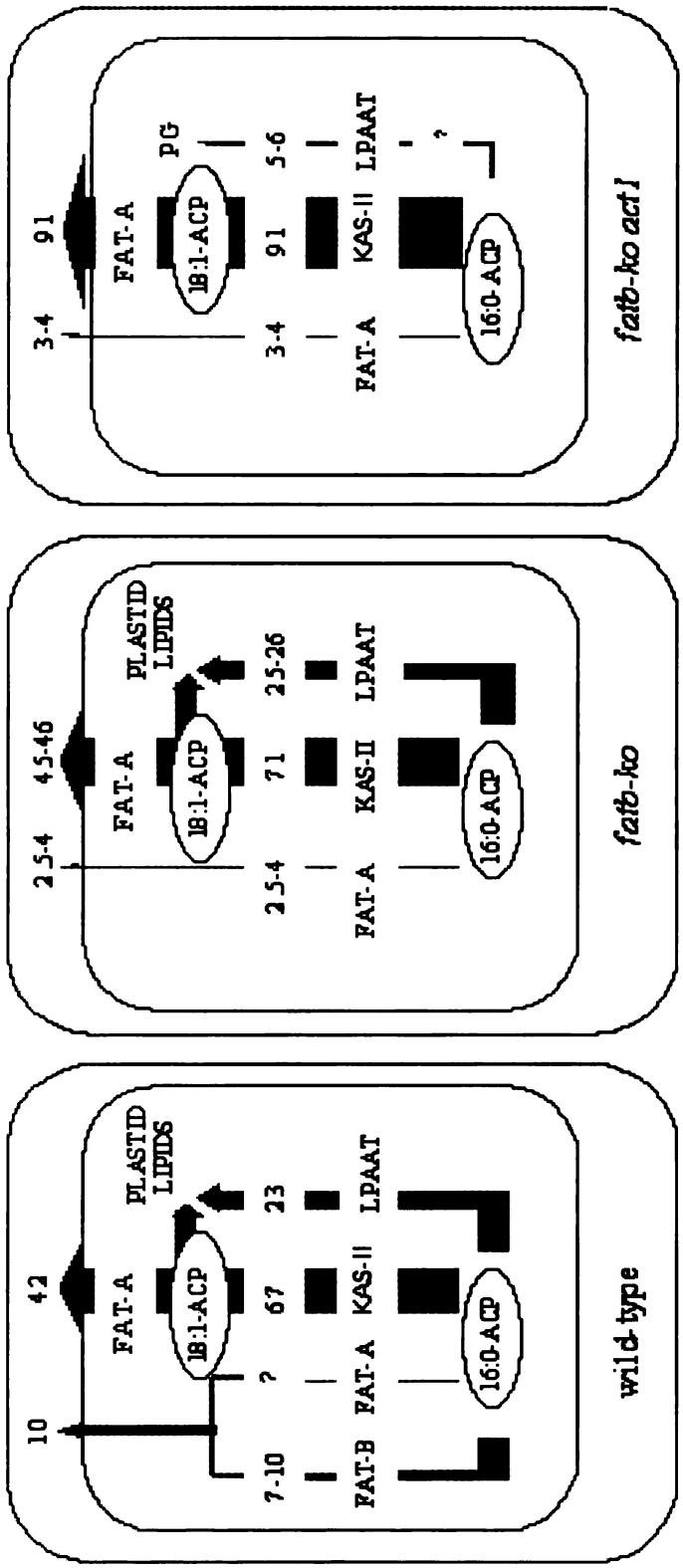
wild-type
fatb-ko
fatb-ko act1
FAT-B
FAT-A
KAS-II
LPAAT
PG
16:0-ACP
18:1-ACP
PLASTID LIPIDS
7-10
10
?
67
42
23
2 5-4
71
45-46
25-26
3-4
91
5-6
?

Figure 25

or its hydroxylated derivatives can be N-linked to sphingoid bases to form ceramides and sphingolipids. Second, in epidermal cells, saturates (16:0 and 18:0) are precursors of the cutin/suberin monomers and wax components. (Post-Beittenmiller,1996). Third, myristoylation (14:0) and palmitoylation (16:0) of proteins is critical for the localization and regulation of protein activity (Yalovsky *et al.*, 1999).

In wild-type leaves the total 16:0 content is about 1.9 μmoles/gfw. Deducting the contribution from prokaryotic lipid synthesis, about 1.2 μmoles/gfw of 16:0 must be exported from the plastid to fuel glycerolipid synthesis. Sphingolipid base synthesis requires another 0.5 μmoles/gfw of palmitate export. In addition, there are significant levels of 16:0 and 2-hydroxy-16:0 N-acyl groups in sphingolipids, so the total flux of palmitate into sphingolipids is actually higher. Thus sphingolipid synthesis may consume 30-40 % of the total cytosolic palmitate pool. However, despite a reduction of about 50 % in extraplastidial 16:0 in the *fatb* mutant, the total amount per fresh weight of sphingoid bases in leaf tissue was similar to wild-type. One interpretation of the constancy of sphingolipid production is that sphingoid base synthesis is tightly maintained at the expense of acyl composition changes in other glycerolipids. Furthermore, because sphingolipids are essential lipids for cell growth (Wells and Lester, 1983) the slow growth of the *fatb-ko* plants could result from a slower supply of the critical 16:0 component to sphingolipids.

Although in leaf mesophyll cells the major fraction of fatty acids is used for biosynthesis of membrane glycerolipids, in epidermal cells most newly produced fatty acids are directed toward the biosynthesis of cutin and epicuticular waxes. In Arabidopsis leaves, an epicuticular wax load of 0.2 μmoles/gfw represents only approximately 10 % of the total leaf pool of palmitate plus stearate, but within the epidermal cells the proportions will be much higher. Analysis of leaf and stem epicuticular waxes in the *fatb-ko* mutant showed a 20 % and 50 % reduction in total wax load respectively, indicating that the FATB thioesterase is one source for production of wax precursors in epidermal cells. Moreover, a reduction of about 85 % in 16 carbon derivatives from cutin demonstrated that FATB supplies palmitic acid for cutin biosynthesis.

In leaf tissue of the *fatb* mutant, all extra-plastidial phospholipids showed a reduction of approximately 50 % in the relative 16:0 levels (Table 9), except PC that showed a 78 % reduction. The fact that PC was most affected may suggest that this phospholipid is a key pool in the delivery or partition of palmitate in the cell, for example as an indirect donor of saturated groups to sphingolipid biosynthesis. In addition, the 10-fold reduction in the relative 18:0 levels also implicated that PC may play a role in 18:0 partitioning. PC plays a major role in the flux of glycerol and fatty acids during membrane glycerolipid biosynthesis and therefore an intriguing question is whether the larger changes in the fatty acid composition of PC could affect its function and be responsible, at least in part, for the slower growth rate of the *fatb*-knockout plants.

Conclusions

The *fatb-ko* line shows a reduction in saturated fatty acids exported to the cytosol, a 17 % reduction in the rate of growth, and altered seed morphology and germination. Although this study clearly demonstrates the requirement for the *FATB* gene and saturated fatty acids for normal rates of Arabidopsis growth and viable seed formation, the specific function(s) supplied by saturated fatty acids to sustain normal growth are still uncertain. Other than the reduction in saturated fatty acid content, and a decrease in wax load, alterations in glycerolipids and sphingoid base compositions were minor. Future work will focus on whether the growth rate of the mutant is linked to the biosynthesis of specific cellular components, subtle variations in membrane properties or changes in FAS/lipid turnover-degradation rates or a combination of effects. The recent development of new isotope labeling techniques to investigate lipid synthesis and turnover-degradation will be critical to answer these questions (Pollard and Ohlrogge, 1999; Bao *et al.*, 2000). However, it is important to note that a *lack* of change of a critical component for growth may point to its essential nature more than change. Thus, the slower production of an essential lipid in the *fatb* mutant could slow growth to a balance point between synthesis of that component and growth and therefore, no change in the level of the key component per plant weight would be expected in the mutant. For example, although we observed no overall reduction in sphingoid base accumulation, the essential nature of sphingolipid synthesis for growth in other systems (Wells and Lester, 1983) suggests compositional changes might not be expected. Similarly, if rates of protein acylation, cutin biosynthesis or synthesis of other saturate derived components are essential to growth, a biochemical phenotype in these components may not be observed. Thus, the isolation of suppressor mutants for the *fatb-ko* phenotype may also provide insights into the underlying

mechanisms which connect the supply of saturated fatty acids to the biosynthesis and/or regulation of essential plant growth components.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* and *fatb-ko* mutant plants (ecotype Wassilewska) were grown at 80-100 μmol m^{-2} s^{-1}, 22°C under 18-h light/6-h dark photoperiod. Seeds were always estratified for three days at 4°C. Selection of T-DNA tagged plants was carried out by soaking the soil with 50 μg/mL of commercial BASTA (Finale) (AgrEvo, Montvale, NJ). Surface sterilized seeds of Arabidopsis were germinated on 0.8 % (w/v) agar solidified MS medium supplemented with 1 % (w/v) sucrose. For the experiments at different temperatures the plants were grown for 2 weeks at 22°C (16 hours light) and then transferred to 16°C and 36°C or kept at 22°C under identical lighting conditions.

Arabidopsis *fatb-ko act1* double mutant was generated by using Arabidopsis *fatb-ko* plants as a pollen donor and Arabidopsis *act1* (Kunst *et al.*, 1988) as pollen recipient. Seeds obtained from the crosses were sown on soil in the presence of 50 μg/mL BASTA in order to select for heterozygous *act1*/+ *fatb-ko*/+ seedlings. F2 seedlings from F1 heterozygous plants were screened by GC and PCR in order to detect homozygous, heterozygous and wild types for *act1* and *fatb-ko* loci.

Mutant Isolation

A transfer DNA (T-DNA, 5.5 Kb) insertion into the *FATB* gene was identified by screening pooled genomic DNA prepared from a T-DNA tagged *Arabidopsis thaliana* (ecotype Wassilewska) collection (Sussman *et al.*, 2000). The gene-specific primers used for the screening of insertions into the *FATB* gene were 5'-CTCATATCCACATATATCTCTCTCTCACC-3' forward and 5'-CAAGCAAGCAAGGTGGTAGTAGCAGATAT-3' reverse, and the T-DNA specific primer matching the left end of the T-DNA (JL-202) was 5'-CATTTTATAATAACGCTGCGGACATCTA C-3'. A *FATB* genomic fragment was labeled using random priming and used to detect specific PCR products by Southern blot hybridization (Sambrook *et al.*, 1989). The T-DNA/*FATB* junctions from both ends of the insertion were PCR amplified, subcloned and sequenced from both ends to determine the insertion point for the 5.5 kb T-DNA.

Complementation Analysis

The binary vector pBINAR-Hyg (Becker D, 1990) carrying the wild-type FATB cDNA was a gift from P. Doermann. The vector was used to transform Arabidopsis *fatb*-knockout plants by *Agrobacterium* vacuum infiltration (Bechtold *et al.*, 1993). The transformation of homozygote plants for the *FATB* T-DNA insertion did not render transgenic seedlings and therefore heterozygote *fatb-ko* plants were transformed. Transgenic seedlings were first selected on agar plates in the presence of 25 μg/mL of hygromycin-B. After 7 days, seedlings were transferred to soil pre-soaked with 50 μg/mL of BASTA in order to select against wild-type plants for the *fatb*-knockout T-DNA

insertion. DNA was extracted from hygromycin-B (transgene T-DNA) and BASTA (knock out T-DNA) resistant plants by using the Qiagen Plant DNA extraction kit (Chatsworth, CA). Homozygote *fatb-ko* plants were identified by PCR using the same primers as for the PCR originally used to isolate the *fatb-ko* mutant.

Real-Time PCR Quantification of mRNAs

Total RNA was prepared from leaf tissue of wild-type (Wassilewska) and *fatb-ko* mutant Arabidopsis by using the Qiagen Plant RNA extraction kit (Chatsworth, CA) according to the instructions. A 5 ug aliquot was used as a template for cDNA synthesis employing the SuperScript First Strand Synthesis system and oligo dT primers (Stratagene). Specific primers for the second and third exons of the *FATB* gene were designed with Primer Express software (PE Applied Biosystems). The sequences of forward and reverse primers are: 5'-AATCATGTTAAGACTGCTGGATTGC-3' and 5'-ATACCATTCTTTCCAGACTGACTGA-3' respectively (Figure 19). Primers were verified by showing that the PCR reaction product produced a single band after agarose gel electrophoresis. Real Time Quantitative PCR analysis was performed according to the manufacturer's instructions (PE Applied Biosystems). The reaction contained, in a final volume of 30 μL, 250 ng of reverse transcribed total RNA, 1.5 μM of the forward and reverse primers, and 2X SYBR Green PCR Master Mix. All reactions were performed in triplicate. The relative amounts of all mRNAs were calculating using the Comparative CT method as described in User Bulletin #2 (PE Applied Biosystems). Arabidopsis eukaryotic protein synthesis initiation factor 4A1 (eIF4A1) mRNA was used as an internal control for variations in amounts of mRNA. Levels of FATB mRNA were

normalized to eIF4A1 mRNA levels and presented as a ratio between wild-type and *fatb-ko* mutant plants. The forward and reverse primers used to amplify eIF4A1 mRNA were 5'-CCAGAAGGCACACAGTTTGATGCA-3' and 5'-AGACTGAGCCTGTTGAATCACATC-3' respectively.

Fatty Acid Analysis of Glycerolipids from Different Tissues of Arabidopsis

Approximately 0.1 g fresh weight (gfw) of tissue from 5-week old Arabidopsis plants was heated at 90°C for 1 hour in 0.3 mL of toluene and 1 mL of 10% (v/v) boron trichlodide/methanol (Sigma) with heptadecanoic acid (17:0) as an internal standard. After acidification with aqueous acetic acid fatty acid methyl esters were extracted two times with hexane and analyzed by gas chromatography with a flame ionization detector (GC-FID) on a DB-23 capillary column.

Individual Glycerolipid Analysis

One gfw of leaf tissue from 5-week old Arabidopsis plants was ground in liquid nitrogen. Lipids were extracted in hexane-isopropanol and glycerolipid classes were separated by thin layer chromatography (TLC) on K6 silica plates (Whatman Inc, Clifton, PA) impregnated with 0.15 M ammonium sulphate and activated for 3 hours at 110° C (Kahn and Williams, 1977). The TLC plates were developed three times with 91:30:8 (v:v:v) acetone:toluene:water and lipids were detected after spraying with 0.2 % (w/v) 2'-7'-dichlorofluorescein/methanol and viewing under UV light. Standards were used to identify the different glycerolipid classes. Lipids were eluted from the silica with

chloroform-methanol and fatty acid methyl esters prepared and analyzed as described above.

Leaf Epicuticular Waxes

Approximately 3 gfw of leaf tissue from 5-week old Arabidopsis plants were used for epicuticular wax analysis. The tissue was dipped in chloroform for 30 seconds and then the following internal standards (IS) were added; n-octacosane at 20 μg per gr of fresh weight, docosanoic acid and 1-tricosanol both at 10 μg per gr of fresh weight. All the compounds were purchased from Sigma. After evaporation of the chloroform under nitrogen the epicuticular waxes were silylated to convert free alcohols and carboxylic acids to their trimethylsilyl(TMS)-ethers and -esters respectively. The epicuticular waxes were heated at 110° C for 10 min in 100 μL of pyridine and 100 μL of N,O-*bis*(trimethylsilyl)trifluoroacetamide (Sigma). After cooling, the solvent was evaporated under nitrogen and the product was resuspended in 1:1 (vol:vol) heptane:toluene for GC analysis. GC conditions were the following: a HP-5 capillary column (30 m x 0.32 mm x 0.25 μm film thickness) with helium carrier gas at 2 ml/min was used; injection was in split mode; injector and FID-detector temperatures were set at 360°C; and the oven temperature was programmed at 150°C for 3 minutes, followed by a 10°C/min ramp to 350°C, and then held for an additional 20 min at 350°C. GC/MS analysis was also performed to identify components of the mixture.

Sphingoid Base Analysis

Approximately 1 gfw of leaf tissue from 5-week old Arabidopsis plants was heated at 110°C for 24 hours with 4 mL of dioxane (Sigma) plus 3.5 mL of 10 % (w/v) aqueous $Ba(OH)_2$ (Sigma) (Sperling *et al.*, 1998). D-erythro-sphingosine (Matreya Inc, Pleasant Gap, PA) at 25 μg/gfw and heptadecanoic acid (Sigma) at 400 μg/gfw were added as internal standards. After saponification the sample was extracted with chloroform to obtain the sphingoid bases in the organic phase and fatty acids in the alkaline aqueous phase. Each phase was independently analyzed as described below.

The chloroform fraction containing the sphingoid bases was back-extracted with an equal volume of 0.4 M aqueous HCl. The acid aqueous phase (containing the protonated sphingoid bases) was then titrated with KOH to pH = 10. The sphingoid bases were re-extracted into chloroform and after evaporation under nitrogen resuspended in 1 mL of methanol. The sphingoid bases were oxidized to their corresponding aldehydes by stirring the sample with 100 μL of 0.2 M sodium periodate (Sigma) at room temperature for 1 hour in the dark (Kojima *et al.*, 1991). The aldehydes were recovered by hexane extraction and used directly for GC analysis. GC conditions were the following: a HP-5 capillary column (30 m x 0.32 mm x 0.25 μm film thickness) with helium carrier gas at 2 ml/min was used; injection was in split mode; injector and FID-detector temperatures were set at 250°C; and the oven temperature was programmed at 100°C for 3 minutes, followed by a 10°C/min ramp to 260°C, and then held for an additional 10 min at 260°C. GC/MS analysis was also performed to identify components of the mixture.

The basic aqueous phase was acidified with HCl to pH < 4 and extracted twice with hexane to recover fatty acids. Fatty acids were transmethylated and analyzed by GC using the same protocol as indicated above.

Monoglucosylceramide (MGC) Analysis

Approximately 10 g of Arabidopsis leaf tissue was quenched with 50 mL of hot isopropanol and ground in a polytron. The extract was filtered and the residue extracted with 25 mL of 2:1 (v:v) chloroform:methanol and re-filtered. This residue was re-extracted with 25 mL of 1:2 (v:v) chloroform:methanol and again filtered. All the three filtrates were combined and evaporated to dryness on a rotary evaporator. The lipid fraction was finally dissolved in 5 mL of chloroform ("Lipid Fraction" in Table 10). The solvent extracted residue dried under vacuum ("Solvent Extracted Residue" in Table 10). The lipid fraction was subjected to a partial base transmethylation to convert most of the O-acyl glycerolipids to fatty acid methyl esters under conditions which leave the N-acyl groups intact. This was achieved by vortexing the lipids (100 mg) with 2M KOH in methanol (0.6 mL) plus hexane (4 mL) for 2 min at room temperature. The reaction was quenched by adding 4 mL of 1M aqueous acetic acid. The hexane phase was removed and the acidified aqueous phase extracted with 2:1 then 1:2 (v:v) chloroform:methanol. The hexane and chloroform:methanol fractions were combined and evaporated to dryness under nitrogen. Lipids were analyzed by TLC as described for "Individual Glycerolipid Analysis" above. A band that co-migrated with beta-D-Glucosyl Ceramide standard (Matreya Inc, Pleasant Gap, PA) was eluted from the silica with 2:1 and 1:2 (v:v) chloroform:methanol. The solvent was dried under nitrogen and the MGC sample was

cleaved by alkaline hydrolysis in 1 mL dioxane (Sigma) and 1 mL 10% (w/v) aqueous $Ba(OH)_2$ (Sigma) for 24 hours at 110°C. Sphingoid bases were recovered and their aldehyde-derivatives analyzed as indicated above for total sphingoid base analysis.

Cutin Analysis

Approximately 20 g of leaf or stem tissue was ground in liquid nitrogen and extracted with 200 mL of isopropanol. The extract was filtered and the residue re-extracted with 200 mL 2/1 (v/v) chloroform/methanol. After filtering the residue was re-extracted with 200 mL 1/2 (v/v) chloroform/methanol and air-dried. An excess (2.5 times by weight) of $LiAlH_4$ plus 6 mL of tetrahydrofuran were added to 100 mg of residue. Hydrolysis was at 80°C for 48 hours with periodic vortexing. After hydrolysis, the excess of $LiAlH_4$ was decomposed by careful addition of ethyl-acetate to the reaction mixture. The mixture was acidified by the addition of 5 mL of water plus 0.8 mL of concentrated HCL. Cutin components were extracted two times with 6 mL of diethyl-ether. The ethereal solution was dried under nitrogen and the sample dissolved in 0.1 mL pyridine plus 0.1 mL of BSTFA (SIGMA). Silylation was at 110° C for 10 min. The excess of reagent was evaporated under nitrogen and the sample dissolved in 1/1 (v/v) heptane/toluene for GLC-MS analysis in an HP-5 capillary column with the oven temperature programmed at 90°C for 5 min, followed by 10°C/min ramp to 300°C, and then held for an additional 10 min at 300°C.

Acyl-ACP Thioesterase Activities

Assays for 16:0-ACP and 18:1-ACP hydrolysis were performed according to Eccleston and Ohlrogge, 1996.

Scanning electron microscopy

Plants were harvested at 3 weeks of age and fixed in 3% glutaraldehyde in 0.1 M PIPES (pH 7.2) overnight at 4°C. Samples were washed three times with 0.1 M PIPES (pH 7.2), 10 min per wash, before dehydration through graded ethanol series (50, 60, 70, 80, 90, 100%). Dehydrated samples were transferred to a drying apparatus and critical point dried at CO_2. Dehydrated specimens were affixed to stubs with double-sided tape and coated with gold in argon. SEM pictures were taken at the Center for Advanced Microscopy at Michigan State University in a JEOL (Japan Electron Optics Laboratories) JSM-6400V scanning electron microscope.

Transmission electron microscopy

For TEM small leaf samples were cut from the middle of the oldest true leaves and immersed in 3%glutaraldehyde in 0.1 M PIPES buffer (pH 7.2). Samples were then fixed overnight at 4°C as described above. After three washes in 0.1 M PIPES buffer, the tissues were postfixed in 2% aqueous osmium tetroxide overnight at 4°C. Samples were again washed 3 times in 0.1M PIPES (pH 7.2) and then dehydrated through graded ethanol series as above. TEM samples were embedded in epoxy resin and thin sectioned before staining.

Accession Numbers

The accession numbers for the *Arabidopsis thaliana* proteins described in Figure 25 and text are: acyl-ACP thioesterase B (FATB) (At1g08510); acyl-ACP thioesterase A (FATA) (At3g25110 and At4g13050), glycerol-3-phosphate acyltransferase (ACT1) (At1g32200), 3-ketoacyl-ACP-synthase-II (KAS-II) (At1g74960), lyso-phosphatidate sn-2 acyltransferase (LPAAT) (At4g30580) and eIF4A1 (At3g13920).

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CHAPTER 4

DISRUPTION OF THE *FATB* GENE INCREASES THE RATE OF FATTY ACID BIOSYNTHESIS,FATTY ACID AND LIPID TURNOVER, AND FAS PROTEIN EXPRESSION IN LEAVES.

ABSTRACT

Disruption of the *FATB* gene in Arabidopsis (*fatb-ko*) results in ~ 60 % reduction in saturated fatty acid export from plastids and 17 % reduction in the growth rate of the mutant compared to wild type (Chapter 3 and Bonaventure *et al.*, 2003). In this study we report that although *fatb-ko* seedlings grow more slowly than wild type the rate of fatty acid synthesis (FAS) in leaves of the mutant increases by 40 %. Moreover, the protein levels of BCCP, ACPs and 18:0-ACP desaturase are increased by 1.5-2 fold in *fatb-ko* leaves compared to wild type, suggesting concerted up-regulation of FAS protein expression. To maintain constant amounts of fatty acids in leaves, thereby counterbalancing their higher rate of production, the mutant also increases by 70 % the initial rate of fatty acid breakdown. However, although *fatb-ko* leaves have higher rates of fatty acid synthesis and turnover, the composition of membrane lipids is similar to wild type. Thus, homeostatic mechanisms to preserve membrane compositions are functional and compensate for substantial changes in rates of fatty acid and glycerolipid metabolism in the mutant The results suggest that *fatb-ko* cells attempt to increase saturated fatty acid production, however a surplus of fatty acids above amounts needed for leaf growth is created and leads to increased turnover. In the mutant, the surplus of fatty acids exported from leaf plastids (largely C_{18} unsaturated fatty acids) and

incorporated into polar lipids is mainly lost via degradation or interconversion of C_{18}:C_{18}-PC species. In addition, C_{16} fatty acids are also rapidly removed from this phospholipid in *fatb-ko* leaves, suggesting that mechanisms are not present which can preferentially preserve the saturated fatty acids.

INTRODUCTION

In plants, the major pathway for the *de novo* fatty acid synthesis occurs in the plastid (Ohlrogge *et al.*, 1979) and this organelle exports fatty acids to supply a diverse array of extraplastidial biosynthetic pathways and cellular processes in the cell (Kolattukudy, 2003; Yalovsky *et al.*, 1999; Post-Beitenmiller, 1996; Lynch, 1993). Production of fatty acids to be exported from plastids depends on the activity of acyl-ACP thioesterases (FATs) that hydrolize the acyl group from acyl-acyl carrier protein (acyl-ACP) to release free fatty acids and ACP (reviewed by Voelker *et al.*, 1997). After export from the plastids, free fatty acids are re-esterified to coenzyme-A (CoA) to form the cytosolic acyl-CoA pool (Pollard and Ohlrogge, 1999). In most plant cells, these molecules are primarily used for the biosynthesis of membrane glycerolipids at the level of the endoplasmic reticulum (ER) (Browse and Somerville, 1991). However, in other tissues exported acyl-CoA molecules have different destinations. For example, in oilseeds, the major fraction of acyl groups is incorporated into triacylglycerols (TAG) that may constitute more than 90 % of total lipids. In epidermal cells, a significant fraction of acyl-CoA molecules is utilized for the synthesis of waxes and cutin (Kolattukudy, 2003; Post-Beittenmiller, 1996). Furthermore, all cells synthesize sphingolipids, and we recently

estimated that as much as 30-40 % of exported 16:0-CoA is needed for sphingoid base synthesis in leaves (Bonaventure *et al.*, 2003). Finally, exported 14:0-CoA and 16:0-CoA participates in other acylation reactions, such as protein acylation (Yalovsky, 1999).

Because acyl-ACP thioesterases terminate fatty acid synthesis and allow the export of fatty acids from plastids, these enzymes are critical regulators of cellular metabolism, controlling the partitioning of *de novo*-synthesized fatty acids between plastids and cytosol and therefore their supply to diverse cellular processes. Two classes of FAT enzymes has been described in most plants, namely FATA and FATB (Voelker *et al.*, 1997). The FATA class has highest in vitro activity for 18:1-ACP and lower for saturated acyl-ACP substrates. In contrast, the FATB class prefers saturated acyl-ACP but also shows activity for unsaturated acyl-ACPs (Salas and Ohlrogge, 2002; Voelker *et al.*, 1997; Doermann *et al.*, 1995). The Arabidopsis genome encodes two FATA genes and a single FATB gene (Beisson *et al.*, 2003; Mekhedov *et al.*, 2000). We previously described the isolation and analysis of an Arabidopsis mutant disrupted in the *FATB* gene (*fatb-ko*) (Bonaventure *et al.*, 2003). In this mutant, the overall amounts of saturated fatty acids in different tissues are reduced by 40 to 50 % compared to wild type. This reduction occurs only in the cytosolic pool of saturated fatty acids, affecting the fatty acid composition of extraplastidial glycerolipids, synthesis of wax in leaves and stems, and the fatty acid composition of TAGs in seeds. However, although sphingolipid synthesis is initiated from 16:0-CoA, no reductions were observed in total sphingoid base content, suggesting that plants may prioritize the synthesis of these essential lipids (Wells and Lester, 1983). These observations also suggested the existence of a metabolic hierarchy

in the economy of saturated fatty acids in plant cells, which prioritizes synthesis of essential components for growth (Bonaventure *et al* 2003). The reduction in the pool of cytosolic saturated fatty acids also slows down the growth of the mutant, resulting in seedlings approximately half the size of wild type seedlings (Bonaventure *et al*, 2003). Thus, reduction in the export of saturated fatty acids from plastids affects cellular processes that are critical for plant growth.

Complete suppression or disruption of any of the core enzymes of FAS would be expected to reduce fatty acid synthesis and affect plant performance. In support of this, analysis of tobacco plants engineered to constitutively express an antisense transcript of the tobacco biotin carboxylase (BC) or the Arabidopsis biotin carboxylase carrier protein (BCCP), showed reductions of leaf fatty acids together with a stunted phenotype (Shintani *et al*, 1997). Growth phenotypes together with reduction in leaf lipids were also obtained with antisense constructs directed against stearoyl-ACP desaturase and ACP4, and with induced mutations in the *fab2* and enoyl-ACP reductase genes (Branen *et al.*, 2003; Mou *et al.*, 2000; Lightner *et al.*, 1994). It is apparent from these studies that even slight reductions in leaf fatty acids synthesis can have pleiotropic effects on plant growth and development. Some of these effects come from changes in chloroplast membrane structure and therefore loss of photosynthetic capability. By contrast, a wide range of mutations in plant fatty acid desaturases, acyltransferases and condensing enzymes demonstrate that the fatty acid composition of plant membranes can be altered considerably with no apparent phenotype under normal growth conditions (Wallis and Browse, 2002; Kunst *et al.*, 1989; Wu *et al.*, 1994). These mutants reveal the plasticity of

lipid metabolism in plants and the capacity of these organisms to adjust to alternative lipid compositions.

Based on these observations, one possible mechanism responsible for the slower growth of *fatb-ko* plants could be a reduced synthesis of fatty acids and therefore a decline in the rate of membrane lipid biosynthesis (Branen *et al.*, 2003; Shintani *et al.*, 1997). Alternatively, slower growth of the mutant may be the result of reduced synthesis of other critical components such as sphingolipids, cutin and waxes or lower rates of acylation reactions. In addition, lipid-derived signaling molecules that affect growth could be affected in the mutant (Nandi *et al.*, 2003). When *fatb-ko* plants are grown at a range of different temperatures, no suppression of the *fatb-ko* growth phenotype is observed, suggesting that changes in the bulk properties of cellular membranes are not the main factor affecting plant performance (Bonaventure *et al.*, 2003). However, more subtle changes in transport and vesiculation cannot be ruled out. Likewise, when the mutant is grown at different relative humidity levels and even in liquid culture, no reversion of the growth phenotype occurs, suggesting that increased water loss through reduced wax content is also not a major factor affecting growth. Similar results are obtained when *fatb-ko* is supplemented with exogenous sugar and in this case its photosynthetic capability appears not to limit its growth (Bonaventure *et al.*, 2003). In addition, the chlorophyll content and the ultrastructure of *fatb-ko* chloroplasts are similar to wild type (Chapter 3, Figure 22)

To further elucidate the role of saturated fatty acids in plant growth and to understand fatty acid partition and FAS regulation in *fatb-ko*, a series of isotope labeling experiments were conducted. Unexpectedly, the rates of both fatty acid synthesis and turnover were higher in *fatb-ko* than wild type leaves. In addition, up-regulation of three proteins involved in FAS was detected, suggesting that higher FAS rates were achieved at least in part by higher FAS protein expression. Thus, *fatb-ko* plants appear to induce a futile cycle of fatty acid production and degradation, perhaps as an attempt to increase saturated fatty acid synthesis.

RESULTS

Rate of fatty acid biosynthesis in leaves of wild type Arabidopsis and *fatb-ko*.

The rate of fatty acid biosynthesis in leaves correlates mainly with the expansion rate of this organ, being higher in younger leaves and reflecting the demand for new membranes to sustain cell division and chloroplast biogenesis (Bao *et al.*, 2000; Browse *et al.*, 1981). It was previously reported that *fab-ko* plants grow slowly resulting in a 50 % decline in their fresh weight compared to wild type (Bonaventure *et al.*, 2003). To evaluate if the disruption of the FATB gene reduces the rate of fatty acid synthesis in leaves, incorporation of labeled precursors was used to assess the *in vivo* rate of this pathway in Arabidopsis wild type and *fatb-ko*. Because isotope incorporation rates can be influenced by internal pools which might differ between mutant and wild type (Nunn *et al.*, 1977; Cronan *et al.*, 1975), three different labeled substrates were tested.

First, the rate of ^{3}H incorporation into fatty acids from $^{3}H_2O$ was measured in leaf tissue of wild type and *fatb-ko*. The results in Table 13 indicate that mutant leaves incorporated ^{3}H into fatty acids at a rate 40 % higher than wild type tissue. The incorporation of ^{3}H was linear for the first two hours of the assay and followed non-linear kinetics thereafter (data not shown). Similar to other systems, a lag phase of 10-15 min for ^{3}H incorporation into fatty acids was observed (data not shown and Browse *et al.*, 1981). Second, leaf tissue of wild type Arabidopsis and *fatb-ko* were incubated with exogenous [^{14}C] acetate as the label substrate. Similar to the results obtained with radiolabeled water, mutant leaves incorporated ^{14}C from acetate into fatty acids at a rate 30 % higher than wild type (Table 13). The kinetic of ^{14}C acetate incorporation into fatty acids was linear for at least 1 hour (data not shown). Finally, intact wild type and mutant seedlings were labeled with $^{14}CO_2$. As shown in Table 13, *fatb-ko* leaves incorporated ^{14}C from CO_2 into fatty acids at a higher rate than wild type (ca. 50 %). In all the experiments, the chlorophyll content per gram fresh weight (gfw) in leaves of the two plant classes was within 5 % of one another (on average: 1.05 ± 0.01 mg/gfw for wild type and 1.07 ± 0.03 mg/gfw for *fatb-ko*).

Table 13. Rates of fatty acid synthesis (FAS) in leaves of Arabidopsis wild type and *fatb-ko* measured by different radiolabeled substrates

Substrate	Wild type*	*fatb-ko**	Increase[1] (%)
$^{3}H_2O$	2801 ± 139	3878 ± 145	40
$^{14}CO_2$	42.0 ± 1.6	63.6 ± 2.9	50
^{14}C acetate	39.0 ± 1.2	50.9 ± 1.4	30

Units are in nmol of ^{3}H or ^{14}C / h / mg Chl incorporated into fatty acids and values represent initial rates of fatty acid synthesis (see Materials and Methods). [1]Percent increase of *fatb-ko* rates versus wild type rates.

In conclusion, by using three different radiolabeled substrates it was demonstrated that *fatb-ko* leaves synthesized fatty acids at an average rate 40 % higher than wild type.

Rate of fatty acid turnover in wild type and *fatb-ko* leaves

We previously reported that leaves from *fatb-ko* plants contained the same amount of fatty acids per gram of fresh weight than wild type (Bonaventure *et al.*, 2003). Therefore, if the FAS rate in *fatb-ko* leaves is on average 40 % higher than wild type, the rate of fatty acid turnover must concomitantly increase to maintain fatty acid amounts constant in this tissue. Therefore, to test this conclusion fatty acid turnover was evaluated by labeling intact seedlings with a 30 min pulse of $^{14}CO_2$ and then determining the level of ^{14}C in fatty acids at different times up to 120 hours (Figure 26).

During the first 6 hours of the chase period, there was net accumulation of ^{14}C into fatty acids of both wild type and mutant (Figure 26). This net accumulation of label most likely reflected the use of ^{14}C labeled carbohydrates as substrates for fatty acid synthesis (Bao *et al.*, 2000). After 6 h of the initial $^{14}CO_2$ pulse, the amount of label in fatty acids from wild type and mutant began to decay (Figure 26). In the 6-120 h period, the radioactivity in fatty acids dropped by 70 % in *fatb-ko* leaves compared to 47 % in wild type leaves (Figure 26). Therefore, wild type and mutant leaves have an average turnover rate of 9 % and 14 % per day respectively (55 % increase in *fatb-ko*). However, as the label in fatty acids decayed following exponential kinetics in the mutant, the average rates did not properly reflect the actual fatty acid turnover rates (Figure 26). Thus, if initial rates were considered instead (6-24 h period), wild type and mutant plants lost

0.39 and 0.67 nmol ^{14}C/mg Chl/hour, respectively (Figure 26). These results demonstrate that the initial rate of fatty acid turnover in the mutant was approximately 70 % higher that in wild type.

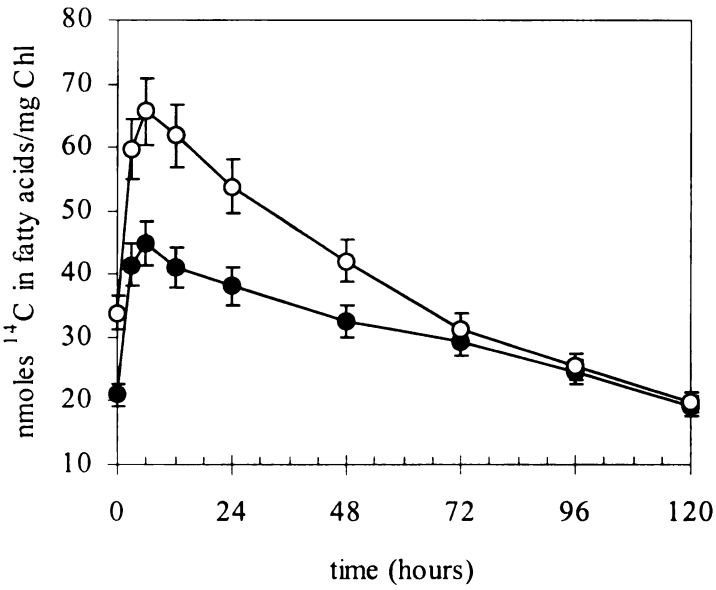

Figure 26. Fatty acid turnover in wild type (●) and *fatb-ko* (○) leaves.

Intact plants were pulsed for 30 min with 2 mCi of $^{14}CO_2$ and fatty acids chased for a period of 5 days in a non-radioactive atmosphere at 22° C. At the times indicated leaf samples were harvested and the fatty acids purified and their radioactivity measured as described in "Materials and Methods".

Analysis of polar lipid classes in wild type and *fatb-ko* leaves

To investigate whether higher rates of fatty acid turnover in *fatb-ko* leaves affected the tunover of particular membrane glycerolipids, we analyzed leaf glycerolipid classes after pulsing wild type and *fatb-ko* seedlings with $^{14}CO_2$ and chasing lipids for different times

up to 120 h. The results shown in Figure 27 indicate that the distribution of radiolabeled plastidial and extraplastidial glycerolipids was similar between wild type and *fatb-ko* leaves.

The label in phosphatidylcholine (PC) from wild type and *fatb-ko* declined by approximately 65 % in the 0-120 h period (Figure 27). This high rate of label disappearance in PC is explained by the donation of diacylglycerol moieties (DAG) from PC to galactolipids and sulfolipids in chloroplasts (Roughan, 1970). Phosphatidylethanolamine (PE) was the only glycerolipid analyzed with lower label in *fatb-ko* than wild type leaves (Figure 27). In wild type tissue, PE accounted for approximately 7 to 9 % of labeled lipids throughout the 5 days period whereas in the mutant represented only 5 to 6 %. Moreover, these levels remained constant throughout the chase period (Figure 27). This observation agreed with the lower steady state amounts of PE observed in *fatb-ko* leaves compared to wild type (Bonaventure *et al.*, 2003). Lower incorporation of radioactivity in PE of the mutant was also observed at early time points during continuous labeling of leaves with [^{14}C]-acetate (data not shown). These data suggest reduced synthesis rather than increased degradation of this phospholipid in *fatb-ko* leaves.

In the case of plastidial lipids, the percentage of label in monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) increased by approximately 25 % and

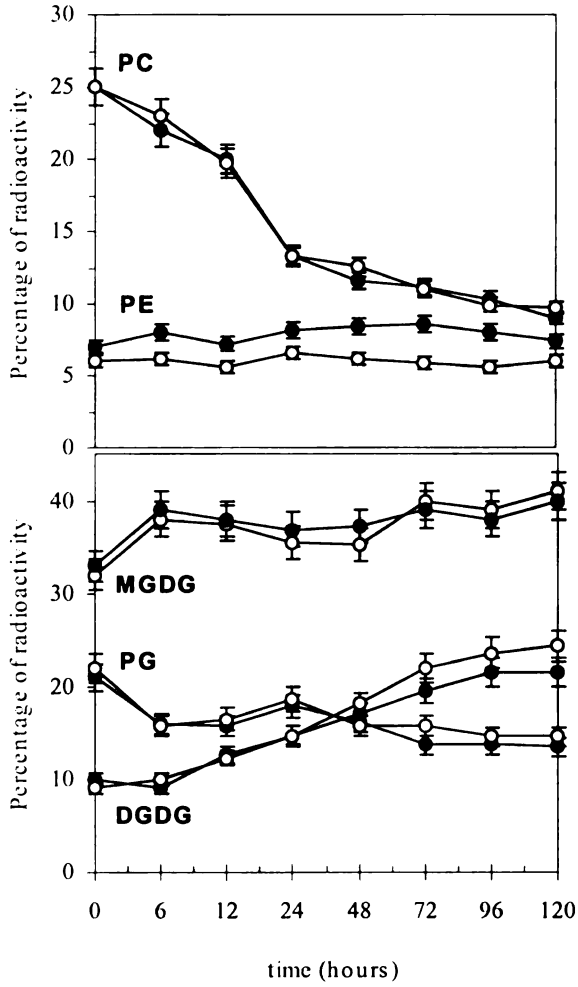

Figure 27. Redistribution of radioactivity among glycerolipid classes of wild type (●) and *fatb-ko* (○) leaves. Seedlings were pulsed with $^{14}CO_2$ and leaf glycerolipids separated by TLC and their activities quantitated as described in "Materials and Methods". Abbreviations: PC, phophatidylcholine; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol.

150 % respectively in both wild type and *fatb-ko* during the chase period (Figure 27). These increments in labeled MGDG and DGDG at longer times represented the contribution of the eukaryotic pathway to the plastidial pool of these lipids (Browse *et al.*,

1989). These data together with the decline of labeled PC are consistent with the parallel operation of the prokaryotic and eukaryotic pathways of lipid biosynthesis in wild type and *fatb-ko* leaves. In contrast, the percentage of label in phosphatidyldiacylglycerol (PG), which is mainly produced in the plastid, declined in both plant classes by approximately 30 % (Figure 27).

The kinetics of ^{14}C labeling in the different lipid classes of wild type Arabidopsis leaves were in accordance with previous studies in which the same tissue was labeled with ^{14}C-acetate (Wu *et al.*, 1994; Browse *et al.*, 1989; Kunst *et al.*, 1989). In conclusion, the similarity in the distribution of label among lipid classes in wild type and *fatb-ko* leaves indicate that despite higher rates of fatty acid turnover cells maintained a constant composition of polar lipids.

Analysis of radiolabeled C_{16} and C_{18} fatty acids in polar lipids of wild type and *fatb-ko* leaves

The fatty acid composition of leaf membrane glycerolipids is different between wild type and *fatb-ko*. In this regard, PE, PS and PI in the mutant have reductions of approximately 50 % in their 16:0 content compared to wild type, whereas in PC the reduction is 80 % (Bonaventure *et al.*, 2003). To understand how the lack of FATB activity affects fatty acid partitioning into leaf glycerolipids, we evaluated redistribution of labeled fatty acids in these lipids. Wild type and mutant seedlings were pulsed with $^{14}CO_2$ and radiolabeled fatty acids in polar lipids chased for different times up to 120 h (Figure 28). In order to obtain an accurate estimate of $^{14}C_{18}$ and $^{14}C_{16}$ levels, fatty acids were first hydrogenated

to remove double bonds and then separated by reverse phase-thin layer chromatography (TLC) according to their chain lengths. The results of the analysis revealed that in the mutant, redistribution of labeled C_{18} and C_{16} fatty acid was similar to wild type in plastidial lipids but differed substantially in extraplastidial lipids (Figure 28).

In PC of wild type leaves, labeled C_{16} fatty acids increased by 25 % in the 0-120 h period (from 20 % to 25 %) whereas in PC of the mutant they decreased by 60 % (from 15 % to 6 %) (Figure 28). These results indicated that in PC of *fatb-ko* leaves, C_{16} fatty acids were first incorporated at 30 % lower levels than wild type and later removed to reach 75 % lower levels than wild type. Moreover, PC in the *fatb-ko* lost almost 50 % of its labeled C_{16} fatty acids in 6 hours, suggesting a rapid removal of this fatty acid from PC (Figure 28). Therefore, the ~80 % reduction in the amount of C_{16} in PC of *fatb-ko* compared to wild type (Bonaventure *et al.*, 2003), results from both reduced incorporation but mainly from higher removal of this fatty acid (Figure 28).

Labeled C_{16} fatty acids in PE redistributed differently from those in PC. In both, wild type and *fatb-ko* leaves their levels decreased after the initial incorporation (Figure 28).

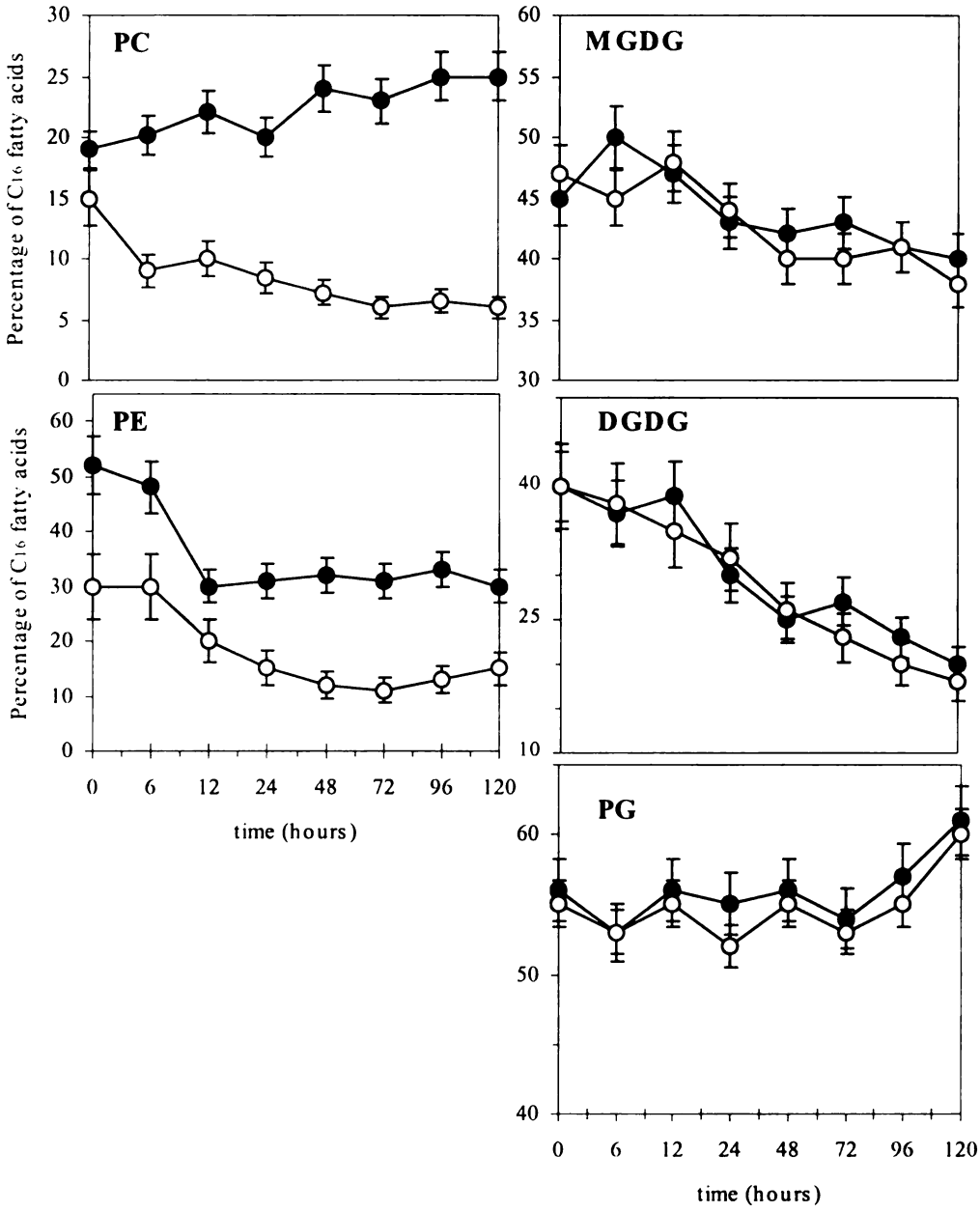

Figure 28. Redistribution of radioactivity in C_{16} fatty acids of membrane glycerolipid classes from wild type (●) and *fatb-ko* (○) leaves. Seedlings were pulsed with $^{14}CO_2$, lipid classes separated by TLC and their corresponding fatty acids transmethylated, hydrogenated and separated by reverse phase TLC. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol.

Nevertheless, PE from mutant leaves incorporated lower levels of C_{16} fatty acids than wild type (Figure 28). Thus, in contrast to PC, the steady state differences in the C_{16} content of PE between wild type and *fatb-ko* was primarily the result of lower incorporation instead of increased removal of this fatty acid (Bonaventure *et al.*, 2003).

In contrast to extraplastidial glycerolipids, the incorporation and redistribution of label in C_{18} and C_{16} fatty acids of plastidial glycerolipids was similar between wild type and *fatb-ko* (Figure 28). These observations agreed with previous studies in which no significant changes in the fatty acid composition of MGDG, DGDG and PG were found between wild type and the mutant (Bonaventure *et al.*, 2003). These results also suggest that the ratio of degradation or interconversion to other components of C_{18} and C_{16} fatty acids from MGDG, DGDG and PG was not altered in *fatb-ko* compared to wild type. In both, MGDG and DGDG, the C_{16} fatty acid levels declined in accordance with their dependence on the prokaryotic and eukaryotic pathways for their synthesis (Figure 28). Moreover, the more pronounced decline of labeled C_{16} in DGDG reflected the higher dependence of this lipid on the eukaryotic pathway (Browse *et al.*, 1986). In the case of phosphatidylglycerol (PG), the situation was different from the other major plastidial lipids and reflected its primary synthesis by the prokaryotic pathway (Browse and Somerville, 1991). The level of C_{16} fatty acids incorporated into this phospholipid at time 0 was 55 % and increased to more than 60 % at 120 h (Figure 28). These data suggest that C_{16} fatty acids in PG were more stable than C_{18} fatty acids.

Mass distribution of C_{18}:C_{18} and C_{16}:C_{18} molecular species of polar lipids in wild type and *fatb-ko* leaves

Arabidopsis membrane glycerolipids are composed of either C_{16}:C_{18} or C_{18}:C_{18} fatty acids in a 1:1 molar ratio. Whereas lipids derived from the prokaryotic pathway are predominantly C_{16}:C_{18}, those derived from the eukaryotic pathway are either C_{18}:C_{18} or C_{16}:C_{18} (Browse and Somerville, 1991). In order to better understand the fluxes of C_{18} and C_{16} fatty acids into lipids and their degradation or interconversion to other lipids, the decay in the mass of labeled fatty acids was calculated for the different molecular species of polar lipids (Figure 29).

The results shown in Figure 29A indicate that in wild type leaves, PC lost 4.8 nmol ^{14}C/mg Chl of C_{18}:C_{18} and 3.3 nmol ^{14}C/mg Chl of C_{16}:C_{18} during the 6-120 h time frame. In parallel, MGDG and DGDG together gained 3.5 nmol ^{14}C/mg Chl of C_{18}:C_{18} and lost 12.4 nmol ^{14}C/mg Chl as C_{16}:C_{18} (Figure 29A). Thus, gain of C_{18}:C_{18} species in MGDG and DGDG could be accounted for by transfer of these molecules from PC. The remaining 1.3 nmol ^{14}C/mg Chl of C_{18}:C_{18} lost from PC could either be degraded in the cytosol or interconverted into other lipid-derived components (e.g., membrane lipids). Thus, in wild type, most of C_{18}:C_{18} loss in PC (73 %) was via MGDG and DGDG biosynthesis in the plastid (Figure 30). The fate of lost C_{16}:C_{18} species in PC could be explained by trafficking of these molecules back to the plastid, degradation in the cytosol or interconversion into other molecules (Figure 30).

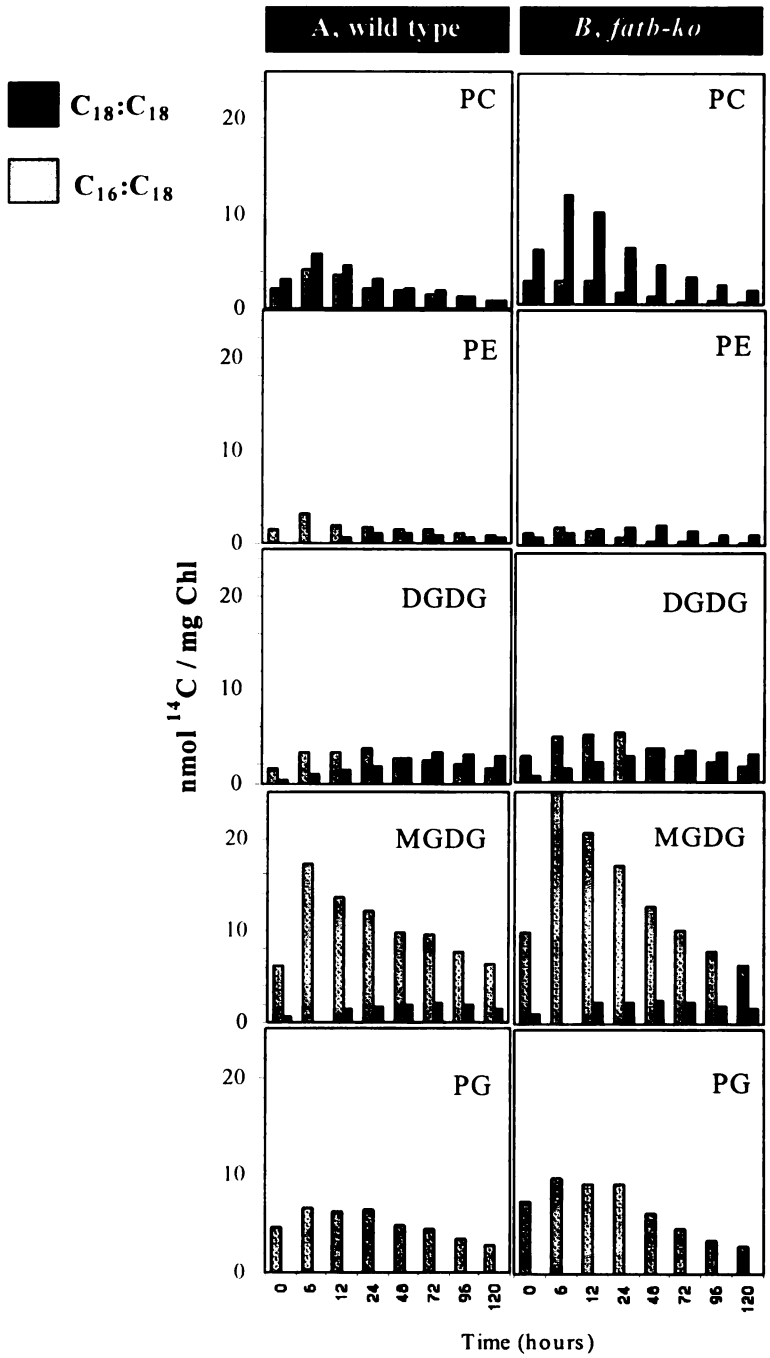

Figure 29. Redistribution of C_{18}: C_{18} and C_{16}:C_{18} molecular species of polar lipids in wild type and *fatb-ko*. Absolute masses for radiolabeled C_{18}: C_{18} and C_{16}:C_{18} species were calculated using total label in fatty acids, polar lipid abundance and percentage of radiolabeled C_{18} and C_{16} in the different polar lipids. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol.

In *fatb-ko* leaves, PC lost 10.4 nmol ^{14}C/mg Chl of C_{18}:C_{18} and 2.4 nmol ^{14}C/mg of C_{16}:C_{18} in the 6-120 h period (Figure 29B). MGDG and DGDG together gained 3.1 nmol ^{14}C/mg Chl of C_{18}:C_{18} and lost 21.9 nmol ^{14}C/mg Chl of C_{16}:C_{18} (Figure 29B). Thus, from the total mass of labeled C_{18}:C_{18} lost from PC only 3.1 nmol ^{14}C/mg Chl contributed to galactolipid synthesis whereas 7.3 nmol ^{14}C/mg Chl were not accounted for. Thus, these results indicate that in contrast to wild type, the major fraction (70 %) of C_{18}:C_{18} lost from PC was not incorporated into galactolipids in the mutant. Importantly, this fraction represented 62 % of the label lost from extraplastidial lipids in *fatb-ko* whereas only 18 % in wild type (Figure 30). The fate of this mass of labeled C_{18}:C_{18} together with lost C_{16}:C_{18} from PC could be degradation in the cytosol, interconversion to other cellular components or trafficking to the plastid in case of the latter (Figure 30).

A second important observation of these experiments was that disappearance of C_{16}:C_{18} species from MGDG, DGDG and PG represented the main loss of mass from labeled fatty acids in wild type and *fatb-ko* leaves. In leaves of the former, total loss of labeled fatty acids was 23.4 nmol ^{14}C/mg Chl in the 6-120 h period (Figures 1 and 5). Disappearance of C_{16}:C_{18} species from MGDG, DGDG and PG accounted for 16.4 nmol ^{14}C/mg Chl, and corresponded to ~70 % of total mass loss (Figure 30). In the mutant, the total loss of labeled fatty acids was 40.8 nmol ^{14}C/mg Chl in the same period and in this case 29 nmol ^{14}C/mg Chl were lost through plastidial C_{16}:C_{18} species (~70 % of total mass loss) (Figure 26 and 5). These results indicate that a major fraction of labeled fatty

acids was eliminated by turnover of C_{16}:C_{18} plastidial species and that the percentage of total label loss by this route was similar between wild type and *fatb-ko* (~70 %).

In summary, these results demonstrate that increased fatty acid synthesis in *fatb-ko* was compensated by increased absolute rates of lipid turnover in a way that preserved the ratio of polar lipids. Furthermore, the results suggest that in the mutant the surplus of fatty acids exported from plastids and incorporated into polar lipids is lost at the level of PC via degradation or interconversion of this phospholipid into other components. In addition, this higher degradation/interconversion rate of PC in *fatb-ko* leaves may explained why C_{16} fatty acids are rapidly removed from this phospholipid and accumulate at levels 80 % lower than wild type, whereas in the rest of the extraplastdial lipids they accumulate at levels 50 % lower than wild type (Bonaventure *et al.*, 2003).

Immunoblot analysis of wild type and *fatb-ko* leaves.

To determine if the increase in the rate of fatty acid biosynthesis in *fatb-ko* leaves was correlated with an up-regulation of FAS protein expression, specific antibodies against BCCP (biotin carboxylase carrier protein), acyl carrier proteins (ACP) and stearoyl-ACP desaturase were used for immunoblot analysis of protein extracts from wild type and *fatb-ko* leaves. As shown in Figure 31 protein levels of the BCCP subunit of plastidic acetyl-CoA carboxylase (ACCase) were increased by 1.5-fold in *fatb-ko* leaves compared to wild type. Similarly to BCCP, the protein levels of ACPs and stearoyl-ACP desaturase were induced by approximately 2-fold in the mutant (Figure 31). Arabidopsis leaves

express several isoforms of plastidic ACPs with ACP-4 as the most abundant in this tissue followed by ACP-2 and 3 (Hlousek-Radojcic *et al.*, 1992). The immunoblot results

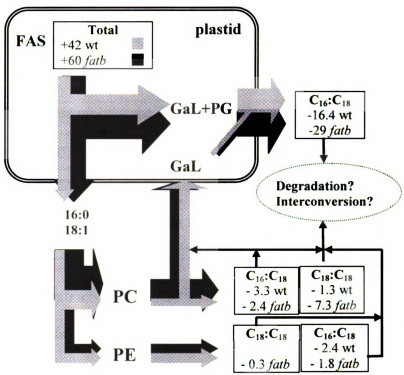

Figure 30. Scheme of mass loss from labeled fatty acids in leaves of wild type and *fatb-ko*. The arrows depict the flux of either fatty acids (16:0/18:1) exported from the plastid or C_{18}:C_{18} and C_{16}:C_{18} in the different lipids (gray: wild type (wt); black: *fatb-ko* (*fatb*)). The values inside the boxes are nmol ^{14}C/mg Chl of C_{18}: C_{18} and C_{16}:C_{18} species. Initial positive values represent total label input at time 6 hours and negative values loss of labeled C_{18}: C_{18} and C_{16}:C_{18} species after 120 hours. Loss of label from galactolipids and PG most likely represent degradation. Loss of labeled C_{16}:C_{18} from PC could not be assigned to a specific pathway, it may occur by conversion to galactolipids, degradation or interconversion to other cellular components. Similar fates can be assigned to lost C_{18}:C_{18}-PC, C_{18}:C_{18}-PE and C_{16}:C_{18}-PE. Abbreviations: GaL: galactolipids (MGDG and DGDG); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol, FAS: fatty acid synthesis pathway.

indicated that most of ACP isoforms were up-regulated in leaves of the mutant (Figure 31). In contrast, a single band in the blot probed with anti-stearol-ACP desaturase antibody appeared up-regulated in *fatb-ko* leaves, suggesting that one major leaf isoform of this enzyme was induced (Figure 31).

Changes in the expression of some FAS proteins occur during leaf development, being higher in young leaves and declining after this tissue completes its expansion (J. Ohlrogge, unpublished results). Thus, to determine whether the increased levels of BCCP, ACPs and 18:0-ACP desaturase in *fatb-ko* were the result of differences in the developmental stage of wild type and mutant leaves, immunoblot analysis was also performed on leaf extracts at different stages of development (2, 3 and 4 weeks old seedlings). The results showed a consistent increase (1.3-2 fold) in the levels of the three FAS proteins in *fatb-ko* leaves compared to wild type at the different stages of development (data not shown).

DISCUSSION

Acyl-ACP thioesterases (FAT) are responsible for the export of fatty acids from the plastid produced by the *de novo* fatty acid synthesis. In this study, isotope labeling experiments and western blots were performed to investigate changes in lipid metabolism and protein expression brought about by disruption of the FATB gene in Arabidopsis (Bonaventure *et al.*, 2003). These experiments suggest that *fatb-ko* alters its fatty acid and

glycerolipid metabolism together with FAS protein expression to compensate for the deficiency in saturated fatty acids. However, higher FAS rate in *fatb-ko* increases the production and export from the plastids of 18:1 over 16:0 fatty acids (Bonaventure *et al.*,

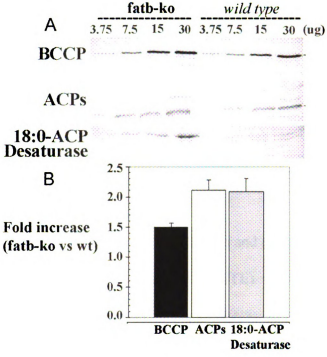

Figure 31. Immunoblot analysis of BCCP, ACP and 18:0-ACP desaturase in leaf tissue of wild type and *fatb-ko* Arabidopsis. A, total Arabidopsis protein was extracted from leaves of wild type and mutant and increasing amounts loaded on the gel (3.75, 7.5, 15 and 30 μg). BCCPs were detected with anti-biotin antibodies, ACPs and 18:0-ACP desaturases with specific antibodies against spinach ACP-I and avocado stearoyl-ACP desaturase, respectively. Relative molecular weight for BCCP-1 is 35 kD, for ACP 10 kD and for 18:0-ACP desaturase 35 kD. When known, major leaf isoforms are noted; B, amount of each polypeptide was quantitated with ImageQuant software and is expressed as relative fold increase of mutant versus wild type. The fold values shown are the average of the fold values for each protein concentration and the bars denote the standard deviation of the average. Fold increase values correspond to BCCP-1, all ACPs detected and major 18:0-ACP desaturase band. The intensity of coomasie-blue stained bands was used to normalize western blot signals.

2003) and the resulting excess of C_{18} unsaturated fatty acids may surpass the capacity of cells to use them for membrane biosynthesis. This surplus leads then to increased fatty acid turnover (Figure 32). Importantly, although *fatb-ko* leaves undergo substantial changes in fatty acid and glycerolipid metabolism, the cells maintain a constant composition of polar lipids. These observations demonstrate the importance of lipid homeostasis in plant cell membranes and how cells adapt glycerolipid metabolism to alternative fatty acid fluxes. Similar results were obtained with the Arabidopsis *act1* mutant in which a major disruption of the prokaryotic pathway for lipid biosynthesis does not affect significantly the lipid composition of the plant (Kunst *et al.*, 1989).

Induction of a futile cycle of fatty acid production and degradation is also observed when the california bay 12:0-ACP thioesterase (MCTE) is over-expressed in developing *Brassica napus* seeds (Eccleston and Ohlrogge, 1998). In this system, the re-direction of fatty acids towards 12:0 reprogram fatty acid metabolism in a way that both the catabolic and biosynthetic pathways are increased to remove the surplus of 12:0 and to maintain normal levels of C_{16} and C_{18} fatty acids, respectively. Thus, although by means of different mechanisms, the missexpression of acyl-ACP thioesterases either in mutant or transgenic plants has major impacts in lipid metabolism and triggers cellular responses to lessen those effects. These observations demonstrate the central role of acyl-ACP thioesterases in balancing and providing fatty acids for several different cellular processes.

Increased rates of fatty acid biosynthesis and turnover in *fatb-ko*

Different labeled substrates have been used to assess the *in vivo* rate of fatty acid biosynthesis in plants, with [1-^{14}C]-acetate, $^{14}CO_2$ and $^{3}H_2O$ the most commonly used (Bao *et al.*, 2000; Pollard and Ohlrogge, 1999; Browse *et al.*, 1981; Jungas, 1968). The advantages of using labeled water over acetate or carbon dioxide to determine *in vivo* rates of product biosynthesis is that water crosses all membranes and rapidly equilibrates with all body water pools (Kelleher, 2001). Moreover, using labeled water eliminates the problem of the dilution of carbon tracers in inaccessible pools and also of different metabolite pool sizes in wild type versus mutant plants (Kelleher, 2001). For example, initial differences in the intracellular pools of acetate between wild type and mutant organisms could result in differential dilution of the tracers inside cells and therefore in different specific radioactivities in labeled fatty acids (Nunn *et al.*, 1977; Cronan *et al.*, 1975). A second important parameter when performing labeling experiments with plants is the use of intact plants versus tissue sections (e.g., leaf discs). A drawback of using the latter is that some artifacts may be introduced by induction of wounding responses that alter fatty acid metabolism (Nishiuchi *et al.*, 1997; Conconi *et al.*, 1996).

Therefore, the observation that three different labeled substrates gave consistent increases in FAS rate of *fatb-ko* compared to wild type, demonstrates that this mutant increases the rate of fatty acid production in leaves. Moreover, the use of cut and intact tissue shows that this effect is independent of differential wounding responses between the mutant and wild type. An important conclusion of these results is that fatty acid production appears not to limit the amount of total membrane lipid biosynthesis and consequently growth of *fatb-ko* plants. Thus, the slower growth of the mutant is most likely the consequence of a

Figure 32. Comparative scheme of fatty acid metabolism in wild type and *fatb-ko.* FAS activation signals are triggered by mechanisms that sense low levels of saturated fatty acids in the cytosol. The same mechanisms may induce the up-regulation of FAS protein expression by transcriptional or post-transcriptional mechanisms. In addition, biochemical and metabolic activation of the FAS machinery are also considered.

Turnover
fatty acids / gfw
18:1 ACP
16:0 ACP
FAS
plastid
wild-type
Turnover
fatty acids / gfw
18:1 ACP
16:0 ACP
FAS
Low levels of saturated fatty acids
?
FAS protein expression
plastid
fatb-ko

Figure 32

reduced supply of saturated fatty acids for cellular processes other than membrane lipid biosynthesis. However, changes in membrane properties as a result of changes in their fatty acids composition can not be ruled out at this point as a mechanism affectting growth of *fatb-ko*.

Wild type and *fatb-ko* leaves presented an average rate of fatty acid turnover of 9 % and 14 % per day respectively (Figure 26). Bao *et al.*, (2000) previously reported a turnover rate of 4-5 % per day in wild type Arabidopsis plants. The higher rate obtained in our experiment may be the result of using different growth conditions and plant ages. In particular, the light:dark period in our experiment was 18:6 hours whereas it was 13.5:10.5 hours in the study by Bao *et al.*, (2000). During light periods the rate of fatty acid turnover is accelerated and therefore longer light periods will increase decay of labeled fatty acids (Bao *et al.*, 2000).

In addition, because *fatb-ko* seedlings have slower growth rate than wild type (Bonaventure *et al.*, 2003) and the decay of labeled fatty acids was followed for a period of 5 days, differences in the net accumulation of fatty acids per seedling were significant between wild type and mutant plants. Wild type seedlings increased by 2-fold their fatty acid content whereas *fatb-ko* seedlings by 1.88-fold in this period of time (data not shown). Thus, the differences between the fatty acid turnover rates of wild type and *fatb-ko* (either 55 % when the average is considered or 70 % when the initial rate is considered) are slightly underestimated.

Turnover of Polar Lipids in wild type and *fatb-ko*

Analysis of the absolute turnover rates of C_{18}:C_{18} and C_{16}:C_{18} molecular species of polar lipids reveal that after 5 days of chase period, approximately 70 % of the label lost in both wild type and *fatb-ko* leaves was as C_{16}:C_{18} species of galactolipids and PG (Figures 4 and 5). This observation agrees with the fact that this pool of lipids contain more than 70 % of the fatty acids in leaves. Moreover, the similar percentage of label lost via galactolipids and PG in wild type and *fatb-ko* further demonstrate the conservation of lipid homeostasis despite higher absolute rates of lipid turnover in the mutant.

One intriguing question is why PC in *fatb-ko* leaves has a bigger reduction in 16:0 content compared to the rest of extraplastidial glycerolipids (Bonaventure *et al.*, 2003). In this study we demonstrate that C_{16} fatty acids are rapidly removed from PC after incorporation in the mutant whereas they accumulate in wild type (Figure 28). One possibility is that the increased turnover of PC in the mutant is not selective for the type of fatty acid and therefore C_{18}:C_{18} as well as C_{16}:C_{18} species of this phospholipid are either degraded or interconverted to other components. Alternatively, more C_{16}:C_{18} species from PC may be used for plastid lipid biosynthesis in the mutant or C_{16} fatty acids selectively removed from PC to supply other cellular processes (e.g., sphingolipid synthesis or acylation reactions).

The kinetics of fatty acid labeling also indicate that the ratio of flux of total fatty acids (C_{18} plus C_{16}) through the prokaryotic and eukaryotic pathways is not substantially

altered in mutant leaves compared to wild type. Hence, the ratio of the distribution of initial masses of ^{14}C-fatty acids into the different polar lipids between these two pathways was similar when wild type and *fatb-ko* were compared (data not shown). Moreover, the data agree with previous results obtained from lipid biochemical analysis (Figure 25 in Chapter 3 and Figure 5 in Bonaventure *et al.*, 2003).

Up-regulation of FAS protein expression

Disruption of the FATB gene in Arabidopsis has provided new insights into some of the regulatory mechanisms that affect expression of FAS enzymes. What are the mechanisms involved in up-regulation of BCCP, ACPs and 18:0-ACP desaturase protein levels in *fatb-ko* leaves? Are there other fatty acid proteins up-regulated in *fatb-ko* leaves? The use of large scale techniques for mRNA profiling will help to investigate whether up-regulation of FAS protein expression occurs at the mRNA level and also to identify additional FAS transcripts with altered expression in mutant versus wild type leaves. Moreover, this analysis could give additional information on changes in the expression of non-lipid genes that may help to understand changes in overall metabolism and perhaps to identify regulatory genes (Ruuska *et al.*, 2002; Girke *et al.*, 2000).

Conclusions and Future Perspectives

The rate of FAS in *fatb-ko* leaves is increased by 40 % compared to wild type and this increment may be partially achieved by up-regulation of FAS protein expression. The

resulting increased supply of fatty acids above amounts needed for leaf growth leads to increased fatty acid turnover. Importantly, despite major changes in fatty acid and glycerolipid metabolisms in the mutant a constant composition of polar lipids is maintained in leaves. The increase in fatty acid synthesis and up-regulation of BCCP, ACPs and 18:0-ACP desaturase protein levels suggest that plant cells have mechanisms capable of sensing subnormal levels of saturated fatty acids and signaling the activation of the FAS machinery and protein expression in order to increase their production. The understanding of the mechanisms responsible for activation of fatty acid synthesis and lipid turnover as well as up-regulation of FAS protein expression in *fatb-ko*, may provide important clues for the understanding of the regulation of fatty acid and lipid synthesis in plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

In all the experiments wild type *Arabidopsis thaliana* and *fatb-ko* mutant plants (ecotype Wassilewska) (Bonaventure *et al.*, 2003) were grown on a mixture of soil:vermiculite (1:1) for three weeks under white fluorescent light (100 μmol m^{-2} s^{-1}) in a 18h:6h light:dark photoperiod at 22°C in growth chambers. Sowed seeds were always stratified for four days at 4°C.

Rate of Fatty Acid Biosynthesis by Arabidopsis Leaves

Rapidly expanding leaves from wild type and *fatb-ko* plants (3 weeks old) were cut in strips (0.5 cm wide) and transferred to pre-weighed glass flasks containing 4.75 mL of incubation buffer (2.5 mM sodium MES (2-[N-Morpholino] ethanesulfonic acid) pH: 5.7, 0.0075 % (w/v) Tween-20 and 2.15 mg/mL of MS salts. Leaf strips from the same plant were randomly distributed in different flasks to ensure sample homogeneity between flasks. After sufficient samples had been prepared (approximately 0.2 g fresh weight per flask), the flasks were re-weighed to obtain gram fresh weight (gfw) values. The assay started by the addition of either 0.25 mL of $^{3}H_20$ (100 mCi/mL, 3.7 GBq/mL) (ARC Inc, American Radiolabeled Chemicals Inc; St. Louis, MO) or 0.025 mCi of [1-^{14}C] sodium acetate (56 mCi/mmol) (ARC Inc, American Radiolabeled Chemicals Inc; St. Louis, MO) to each flask and incubating them for different times at 22°C in a temperature-controlled water bath with gentle agitation and continues illumination. All data points were performed in duplicate and the values presented in Table 13 represent initial rates of fatty acid synthesis (calculated using data from 0, 10, 20, 40, 60 min of continuous labeling). At the end of the assay period the incubation medium was removed and the tissue quickly washed twice with de-ionized water and quenched by heating in 10 mL of isopropanol for 10 min at 80°C. Lipids were extracted with hexane-isopropanol method (Hara and Radin, 1978). An aliquot of the lipid extract was suspended in acetone:water (4:1, v/v) to determine chlorophyll content (Arnon, 1949). The methodology used to determine the rate of fatty acid biosynthesis using $^{14}CO_2$ was identical to the methodology used to determine the rate of fatty acid turnover (see below) with the only difference that plants were removed at different time points from the sealed bag using a double sealed air trap.

Rate of Fatty Acid Turnover by Arabiodpsis Leaves

Wild type and *fatb-ko* plants were grown for 3 weeks as indicated above. One day prior to the experiment, a total of 12 pots (6 pots with wild type and 6 with *fatb-ko* plants (15 plants per pot)) were randomly placed inside a transparent glove bag (40 litre gas space, $I^2R^{®}$ Instruments for Research and Industry, Cheltenham, PA) with circulating air and same lighting and temperature conditions as indicated above. A 30 min pulse of $^{14}CO_2$ was given to the plants by mixing 2 mCi of ^{14}C-$NaHCO_3$ (56 mCi/mmol) (ARC Inc; St. Louis, MO) with concentrated sulfuric acid inside the sealed bag and air circulated by using a small battery-driven fan. For the chase period, the radioactive atmosphere was rapidly vented and the plants were placed in a normal (non-radioactive) atmosphere for different times in the same growth conditions as described above. At each time point , 15 wild type and 15 *fatb-ko* plants were randomly removed from different pots and separated in two individual samples (7-8 plants/sample for wild type and mutant). Leaf tissue was immediately weighed, frozen in liquid nitrogen and stored at -80°C for subsequent lipid extraction with hexane-isopropanol method (Hara and Radin, 1978) and for chlorophyll content determination (Arnon, 1949).

Fatty Acid Analysis of Lipid Extracts from Leaf Tissue

Transmethylation of fatty acids from total lipid extracts was performed similarly for all the experiments. An aliquot of lipid extracts was heated at 90°C for 1 hour in 1 mL of

10% (v/v) boron-trichloride/methanol (Sigma). After acidification with aqueous acetic acid, fatty acid methyl esters (FAMES) were extracted two times with hexane and radioactivity in the sample (either ^{3}H or ^{14}C) analyzed by scintillation counting (Beckman Instruments Inc, Fullerton, CA). A second aliquot of total lipid extract from each sample was transmethylated and FAMES were separated by thin layer chromatography (TLC) on K6 silica plates (Whatman Inc, Clifton, PA) using 90/10 (v/v) hexane/diethyl-ether. Radioactive bands corresponding to FAMES were localized by scanning in an Instant Imager system (Packard, Meriden, CT). The bands corresponding to FAMES were recovered from the plates and radioactivity measured by scintillation counting (Beckman Instruments Inc, Fullerton, CA).

Individual Glycerolipid Analysis.

Glycerolipid classes from total lipid extracts were separated by thin layer chromatography (TLC) on K6 silica plates (Whatman Inc, Clifton, PA) impregnated with 0.15 M ammonium sulphate and activated for 3 hours at 110° C (Kahn and Williams, 1977). The TLC plates were developed three times with 91/30/8 (v/v/v) acetone:toluene:water and scanned for radioactivity using an Instant Imager (Packard, Meriden, CT), both to quantitate radioactivity and to locate the appropiate bands for recovery. Lipids were also detected after spraying with 0.2 % (w/v) 2'-7'-dichlorofluorescein/methanol and viewing under UV light. Standards were used to identify the different glycerolipid classes. Lipids were eluted from the silica with chloroform/methanol/water (5/5/1) and fatty acid methyl esters prepared as described

above. FAMES from the different lipid classes were hydrogenated using hydrogen at slightly greater than atmospheric pressure with a platinum (IV) oxide catalyst in methanol. The reaction was performed for at least 2 hours giving complete reduction of unsaturated to saturated FAMES. Analysis and isolation of 18 and 16 carbon FAMES were by KC18 reverse phase TLC (Whatman, Clifton, PA), developed half and then fully with acetonitrile/methanol/water (130/70/1, v/v/v). Plates were scanned for radioactivity using an Instant Imager (Packard, Meriden, CT) to quantitate radioactivity and to locate the appropiate bands for recovery. Scintillation counting was also used to measure radioactivity after scrapping-off the bands corresponding to 16 and 18 carbon fatty acids from the plates.

SDS-PAGE and Immunoblotting

Extraction of proteins from leaves was performed as follows. Up to 0.1 g of leaf tissue was harvested and placed into a 1.5 mL plastic microcentrifuge tube. Plant material was pulverized with a microcentrifuge pestle in the presence of liquid nitrogen. Powder was immediately reconstituted in 0.2 mL of extraction buffer (2 % (v/v) 2-mercaptoethanol, 50 mM HEPES (pH: 7.8), 100 mM NaCl, 0.05 % (w/v) SDS) by vortexing. Insoluble debris was collected by centrifugation for 15 min at 12,000g. Supernatant was removed and placed into a fresh microcentrifuge tube. Protein concentration was determined by dye-binding protein assay using bovine serum albumin as the standard (Bradford, 1976).

SDS-PAGE and protein transfer to nitrocellulose was performed using standard conditions. Nitrocellulose membranes were blocked for at least 1 h with 10 mM Tris-HCl, pH: 8.0, 0.15 M sodium chloride, 0.3 % (v/v) Tween 20 (TBS-T), and 2 % (w/v) nonfat dry milk. Anti-biotin antibodies conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were directly added at a 1:5,000 dilution to detect BCCP. Antisera raised against avocado stearoyl-ACP desaturase (Shanklin and Somerville, 1991) and spinach acyl carrier protein-I (ACP-I) (Post-Beittenmiller *et al.*, 1991) were added at a 1:1,000 dilution. Probing proceeded for 16 h at 4° C followed by 1 h at 25° C after which membranes were briefly rinsed with TBS-T. Antibody bound proteins were detected by incubating blots for 1 h at 25° C with alkaline-phosphatase-conjugated anti-rabbit secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The membranes were then washed 6 times for 5 min each with TBS-T. Blots were then washed 5 min with developing solution (0.1 M Tris-HCl, pH: 9.5, 0.1 M sodium chloride and 5 mM magnesium chloride) before colorimetric detection in developing solution containing 0.33 mg/mL p-nitro blue tetrazolium chloride and 0.17 mg/mL 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt. Immunoblot signals were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA), which accounted for band area plus intensity. Blot signals were corrected for differences in protein loading (as determined by Coomasie Brilliant Blue R 250 staining) and normalized against values of the control plants. Increasing amounts of total protein were resolved by SDS-PAGE for immunoblot analyses to ensure that both major and minor bands were within the linear sensitivity range of the detection system.

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Chapter 5

FINAL CONCLUSIONS, PERSPECTIVES AND FUTURE DIRECTIONS

The work presented herein used several approaches to increase our understanding of the signals and regulatory mechanisms that influence fatty acid biosynthesis (FAS) in plants. These approaches range from gene expression analysis to detailed biochemical analysis of lipid metabolism in a mutant and the results have provided new insights into regulation of this primary metabolic pathway. In addition, work has focused on the partition of fatty acids from their source inside plastids to an array of biosynthetic pathways outside this organelle. One goal of plant biotechnology is to use these organisms as large scale chemical factories for production of edible and industrial products and as an alternative to petroleum-derived products (Thelen and Ohlrogge, 2002). The understanding of the regulatory mechanisms of fatty acid synthesis and partitioning should contribute to the development of better strategies to manipulate fatty acid production in plants.

Conclusions and future directions on FAS gene expression in Arabidopsis

Major conclusions of this thesis research related to FAS gene expression are:

1. Different environmental and metabolic signals regulate ACP expression in Arabidopsis depending on the ACP isoforms and their tissue specific expression.

2. Up-regulation of a subset of fatty acid and lipid genes and the steady expression of others appear to be sufficient to match new requirements for fatty acids in plant tissues.
3. Mechanisms that sense low levels of saturated fatty acids may signal the up-regulation of FAS proteins in Arabidopsis leaves.

In Chapter 2, the genes encoding for acyl carrier proteins (ACPs) were selected for the initial experiments and results presented in that chapter demonstrate that regulation of these genes depends on the protein isoforms and their tissue specific expression. For example, ACP4 is the most abundant isoform in Arabidopsis leaves and it is regulated by light and carbon, similarly to photosynthetic genes. By contrast, the less abundant leaf isoforms ACP2 and ACP3 are not light-regulated but instead respond to growth, suggesting a more critical role for them in rapidly dividing tissue (e.g., meristems).

One obvious question that arises from the analysis of ACP expression is whether ACP4 is the only fatty acid gene regulated by light in Arabidopsis. A recent report based on GeneChip analysis of phytochrome mutants and different lighting conditions indicates that at least 4 additional genes involved in fatty acid and lipid synthesis respond also to light (Tepperman *et al.*, 2001). These genes include ACP4, chloroplast linoleate desaturase (FAD7), omega-3-desaturase (FAD3), chloroplast omega-6 desaturase (FAD6) and stearoyl-ACP desaturase. Since the microarrays used in that study covered approximately one third of the predicted genes in the Arabidopsis genome (Affymetrix 8K GeneChip), additional fatty acid and lipid genes may also be light regulated.

Are ACP2 and ACP3 the only fatty acid genes that are growth/cell cycle regulated in Arabidopsis? A recent report describes a GeneChip analysis of synchronized Arabidopsis cell suspension cultures used to identify cell-cycle controlled genes (Menges *et al.*, 2002). The results indicate that only ACP2 and 3, two putative acyl-CoA synthetases and chloroplast omega-3-desaturase (FAD7-8) are under cell-cycle regulation in Arabidopsis. Again, only one third of the Arabidopsis genome was covered by the GeneChip used in that study and therefore the list of fatty acid and lipid genes regulated by cell-cycle may extend in the future.

When an Arabidopsis cDNA microarray representing 3,500 genes was used to examine changes in gene expression during Arabidopsis seed development and in particular during the phase of oil accumulation (8-12 days after flowering), it was found that only a subset of genes involved in fatty acid and lipid metabolism showed significant changes in expression (Ruuska *et al.*, 2002). For example, ACCase subunits, KAS I and II, ACP1, and FAD2 and 3 show peaks of expression between 8-12 days after flowering.

In summary, it appears from these observations that rather than up-regulating all or most genes for fatty acid and lipid synthesis, plant cells up-regulate a limited number of them according to the tissue and its specific needs for fatty acids and lipids. Thus, instead of "global" regulatory mechanisms for all or most FAS genes, up-regulation of a subset of them and the steady expression of others seem to be sufficient to match new requirements for fatty acids in different tissue or under changing environmental conditions. Although

we focused our research on ACP genes, a logical extension can be applied to other fatty acid genes and we speculate that most likely all of them respond differentially to diverse signals and can be up-regulated according to tissue requirements (Menges *et al.* 2002, Ruuska *et al.*, 2003; Tepperman *et al.*, 2001). In Arabidopsis, most enzymes involved in fatty acid and lipid synthesis are encoded by multiple genes, and one intriguing question has been why Arabidopsis requires so many different FAS enzyme isoforms (Beisson *et al.*, 2002). Their differential tissue expression and response to signals under variable conditions sheds some light on this question.

Analysis of gene expression in *fatb-ko* leaves, indicates that the protein levels of BCCP, ACPs and 18:0-ACP desaturase are increased between 1.5 to 2-fold in young leaves. Thus, these results suggest that mechanisms that sense low levels of saturated fatty acids can increase the expression of at least some FAS proteins. Therefore a new signal can be added to the repertoire of signals that, by still unknown mechanisms, activate fatty acid protein expression. In addition, it remains to be tested whether additional fatty acid gene products also respond to this regulatory mechanism. Thus, is there a global up-regulation of fatty acid proteins in *fatb-ko* leaves or, are only a subset of critical genes up-regulated? An answer to this question will provide interesting information to correlate induction of specific FAS genes with increased rates of fatty acid production in leaves.

Future directions

Although most of the structural genes for fatty acid and lipid biosynthesis are known or have strong putative candidates, information about regulatory factors for this metabolic

pathway is still poor (Beisson *et al.*, 2003; Mekhedov *et al.*, 2000). Thus, one breakthrough step in plant fatty acid and lipid research will be to isolate regulatory factors for the expression of FAS proteins. One important consequence of the identification of these factors would be to manipulate lipid metabolism in genetically engineered plants. The advantage of expressing regulatory factors rather than single enzymes is that an entire metabolic pathway could be up-regulated and for example increase the yield of desired products (Gantet and Memelink, 2002; Memelink *et al.*, 2001; Grotewold *et al.*, 1998). Nevertheless, one difficulty presented by primary metabolism is that it often is not very amenable to metabolic engineering, in part because it operates in parallel with multiple pathways and therefore has complex interconnections with them (Carman and Henry, 1999). For example, carbon and nitrogen metabolism are closely interconnected and genes involved in nitrogen metabolism are regulated by carbon (Oliveira and Coruzzi, 1999). In addition, alteration of primary metabolism can cause unwanted secondary effects that have to be controlled before engineered plants can be used for commercial purposes (e.g., deformed morphology and size of seeds with reduced saturated fatty acids, Chapter 3-Figure 20). Thus, in order to successfully manipulate fatty acid metabolism in plants, it is helpful to know and consider the complex interconnections between metabolic pathways and their regulation.

The studies performed in Chapter 2 open the possibility to identify transcription factors involved in regulation of fatty acid gene expression. One procedure to achieve this goal is to develop a system in which signal-specific (e.g., growth) changes in gene expression can be followed to perform functional promoter analysis (Sun *et al.*, 2003; Chen *et al.*,

2002; Fujiki *et al.*, 2000). Thus, in Chapter 2 (Figure 15) a system to identify elements involved in expression and regulation of the Arabidopsis ACP2 promoter by growth was developed. Further characterization (deletions and site-specific mutations) of this promoter in tobacco BY-2 cells will allow the identification of short DNA elements important for growth-regulation of this gene. Once these elements have been identified, the isolation of transcription factors can be approached by techniques such as yeast one-hybrid system, oligo-affinity chromatography, and electromobility shift assays (EMSA) (Carles *et al.*, 2002; Uno *et al.*, 2000; Jarrett, 1993). Keeping in mind our longer-term interest in increasing oil production in seeds, one evident question is whether the isolation of factors involved in regulation of ACP2 by growth will also be important for expression of ACP2 or fatty acid genes in seeds. On the one hand, the same transcription factor could be expressed in different tissues and have similar functions perhaps by association with alternative partners. On the other hand, completely different regulatory factors may be used in different tissues. In this case however, the ectopic expression in seeds of, for example, a meristem specific factor, could still have effects on fatty acid metabolism in seeds.

Similarly, a functional promoter analysis of *ACP4* can allow the identification of transcription factors involved in light-regulation of this gene. For this purpose an ACP4 promoter deletion series fused to luciferase can be created and analyzed in leaf tissue of transgenic plants. In parallel, the same plants can be used for the analysis of mechanisms involved in sugar-repression of the *ACP4* gene.

What are the mechanisms involved in up-regulation of BCCP, ACPs and 18:0-ACP desaturase protein levels in fatb-ko leaves? Is this up-regulation at the level of transcription or at downstream processes such as protein turnover? Are there other fatty acid genes up-regulated in fatb-ko leaves?

One experiment that may provide important information about increases in mRNA abundance of BCCP, ACPs and 18:0-ACP desaturase together with additional fatty acid genes is to compare transcript profiles between *fatb-ko* and wild type leaves by microarray analysis. Moreover, this analysis could give additional information on changes in the expression of non-lipid genes that may help to understand changes in overall metabolism and perhaps to identify regulatory genes. One limitation of microarrays is that this technique only searches for differences in transcript abundance and information on translational control or protein degradation are not considered. In addition, transcript abundance for regulatory proteins is usually low and difficult to detect by this method (Vainrub and Pettitt, 2003; Watson and Akil, 2002). Thus, additional experiments will be required to obtain a more complete picture of changes in fatty acid gene expression including translational control (e.g., polysome association) and protein turnover.

What is the role of the CTCCGCC box and polypyrimide tracts in the 5' leader of ACP mRNAs?

The results in Chapter 2 (Figure 9) indicate that the 5'UTR of ACPs have an active role on gene expression. One possible scenario is that specific factors bind to the leader and facilitate the interaction between ribosomes and ACP transcripts. Alternatively, a tertiary

structure in the RNA may form to facilitate ribosome association and presumably translation initiation (Bolle *et al.*, 1996; Staub and Maliga, 1994; Danon and Mayfield, 1991). Several approaches can be followed to answer these questions, for example the conserved elements in the ACP leaders can be replaced with random sequences to test how the association with polysomes and expression of a reporter gene are affected in transgenic plants. Second, the binding capacity of factors present in cytosolic fractions to ACP 5'UTRs can be investigated by electromobility-shift assays (EMSA) (Danon and Mayfield, 1991). Transcript stability can be analyzed by techniques such as mRNA turnover after inhibiting RNA Pol II activity with α-amanitin in seedlings grown in liquid culture (Hua *et al.*, 2001).

Conclusions and future directions on fatty acid metabolism and its regulation in *fatb-ko.*

Major conclusions of this thesis research related to the role of saturated fatty acid are:

1. FATB is a major regulator of saturated fatty acid production in all tissues.
2. Saturated fatty acids have a critical role in plant growth and development.
3. Low levels of saturated fatty acids in the cytosol triggers signals to increase the rate of fatty acid biosynthesis in leaf plastids.

Chapter 3 investigates the production and partition of saturated fatty acids from plastids to other cellular pathways. Analysis of the *fatb-ko* mutant revealed that FATB is a major enzyme responsible for export of saturated fatty acids from plastids in all tissues. Moreover, reduction of cytosolic saturated fatty acid pools brought about by the lack of

FATB caused changes in the distribution of saturated fatty acids between different pathways that reveal a hierarchy in the metabolism of these molecules. For example, although there is more than a 50 % reduction in exported saturated fatty acids in the mutant, sphingoid base amounts in leaves were similar to wild type. In contrast, changes in wax amounts could be detected between these two plant classes. These observations are interpreted to indicate that maintaining sphingolipid levels is more essential than maintaining wax amounts for the plant. Some of the changes in saturated fatty acid-derived pathways have clearly negative effects in plant performance and seed development at standard growth conditions. Thus, saturated fatty acids have a critical role in plant growth and development and the understanding of the cellular steps affected will be important to manipulate fatty acid composition in plants without affecting their performance (see below).

Analysis of fatty acid metabolism in *fatb-ko* also gave new insights into the mechanisms and signals that increase the rate fatty acid biosynthesis in plant cells (Chapter 4). Low levels of saturated fatty acids in the cytosol not only triggers signals to up-regulate fatty acid protein expression but also to increase the rate of fatty acid biosynthesis. These results demonstrate that plants can uncouple rate of growth from rate of fatty acid production and therefore they have the inherent capacity to increase fatty acid production although growing at lower rates than normal. This is an important observation because it suggests that cells can be programmed to produce more fatty acids independent of a developmental program or rate of growth that sets the amount of fatty acids to be made.

Future directions

What signal(s) activate fatty acid synthesis in plants? How is the rate of fatty acid synthesis increased?

These are crucial questions that if answered will give important insights in the regulation of fatty acid biosynthesis in plants. This thesis research suggests that *fatb-ko* provides a new system to tackle these questions. The rate of fatty acid biosynthesis in *fatb-ko* leaves is increased by approximately 40 % compared to wild type and unraveling the underlying activation mechanisms could provide a breakthrough in understanding regulation of fatty acid biosynthesis. One critical issue to begin to answer these questions is to understand where in the cell the signal(s) to activate FAS are being generated. In Chapter 4, several mechanisms have been proposed to explain possible signal sources such as sensing of extraplastidial acyl-CoA pools, rate of sphingoid base synthesis, rate of protein acylation, changes in membrane properties that affect transport or vesiculation. One possible approach to identify the actual mechanisms is to isolate suppressors of *fatb-ko* (see below).

What is the target of these activation mechanisms in fatb-ko leaves? Is ACCase activity altered in fatb-ko leaves?

Several lines of evidence suggest that acetyl-CoA carboxylase (ACCase) is a major regulatory enzyme in fatty acid production in plants and therefore it could be one target of the activation mechanisms (Ohlrogge and Jaworski, 1997). The β-CT subunit of ACCase is phosphorylated and this event is correlated with activation of its activity (Savage and Ohlrogge, 1999). Thus, analysis of the phosphorylation status of β-CT in

fatb-ko and wild type leaves could provide information on ACCase as a target of the activation mechanism induced in the mutant.

In addition, evidence for a negative feedback regulatory mechanism acting on ACCase comes from experiments where tobacco cells were incubated in the presence of exogenous fatty acids (Shintani and Ohlrogge, 1995). Could ACCase in *fatb-ko* leaves be released from some sort of feedback control? It has been proposed that in bacteria ACCase is inhibited by acyl-CoA products, and this inhibition can be released by overexpression of acyl-CoA thioesterases (Jiang and Cronan, 1994). However, plant ACCase appears to be non responsive to long acyl-ACP products in vitro (Roesler *et al.*, 1996). Other potential feedback inhibitors of FAS enzymes could be acyl-CoA, free fatty acids and glycerolipids. Because FAS occurs inside the plastids but the major utilization of the products of fatty acid synthesis is at the ER membranes, it is possible that feedback regulation requires communication across the plastid envelop. Could cytosolic saturated fatty acids be feedback controllers of FAS synthesis?

What is limiting growth of the fatb-ko mutant?

Different hypothesis can be proposed to explain the reduced growth of *fatb-ko* mutant as outlined in Chapter 3. However, pleiotropic effects brought about by the genetic lession could be difficult to interpret and one approach to study this complex system is to identify suppresors of the growth phenotype of *fatb-ko*. The mutant grows slower than the wild type plants and identification of seedlings that performed better than the mutant could be identified in mutageneized *fatb-ko* populations. Mutagenesis of *fatb-ko* can be

performed chemically by using EMS or by T-DNA activation tagging. One advantage of using EMS is that it creates point mutations that could lead to amino acid substitutions and change protein properties without killing its activity (Jander *et al.*, 2003; Mc Callum *et al.*, 2000). On the other hand, activation tagging finds suppressors by overexpression of targeted genes and therefore a different group of genes from those targeted by EMS is selected (Memelink, 2003; van der Fits *et al.*, 2001). Suppresors of *fatb-ko* may fall in different categories, for example genes that increase saturated fatty acids levels, genes involved in biochemical pathways different from FAS, genes involved in regulation of FAS, genes that participate in membrane transport or vesiculation, and changes in protein acylation or properties of acylated proteins. Thus, as mentioned above the suppressor approach could help to unravel both: mechanisms that limit *fatb-ko* growth as well as regulatory mechanisms of fatty acid synthesis. For instance, if a particular cellular process is affected and limits growth, a mutation that bypass this limitation could reside in a regulatory protein of an interdependent process. To illustrate this idea, suppressor mutants of yeast deficient in Golgi vesicle formation were mapped in genes corresponding to transcription factors (HAC1) and kinases (IRE1) that are involved in the unfolded protein response (UPR) signaling pathway (Higashio and Kohno, 2002). Thus, activation of UPR indirectly stimulates ER to Golgi vesiculation and reconstitutes growth of yeast affected in this process. In a different experiment, yeast suppressors of sphingolipid biosynthesis where isolated and named SLC (sphingolipid compensation) (Lester *et al.*, 1993). These strains are viable without synthesizing sphingoid bases and therefore lack ceramides. SLC mutants cells synthesize novel PI (phosphatidylinositol) substituted with long (26 carbons) fatty acids in the *sn-2* position of glycerol. Thus, the

SLC suppressors overcome the essential function of sphingolipids by producing novel PI that structurally mimics sphingolipids.

In conclusion, the identification of regulatory factors and the unraveling of the regulatory mechanisms responsible for FAS activation in *fatb-ko* together with the origin and nature of the inducing signal(s) may provide essential clues for the understanding of the regulation of fatty acid and lipid synthesis in plants.

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