PALMITOYL-ACYL CARRIER PROTEIN THIOESTERASE IN COTTON

(*GOSSYPIUM HIRSUTUM* L.): BIOCHEMICAL AND MOLECULAR

CHARACTERIZATION OF A MAJOR MECHANISM FOR THE REGULATION

OF PALMITIC ACID CONTENT

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The relatively high level of palmitic acid (22 mol%) in cottonseeds may be due in part to the activity of a palmitoyl-acyl carrier protein (ACP) thioesterase (PATE). In embryo extracts, PATE activity was highest at the maximum rate of reserve accumulation (oil and protein). The cotton *FatB* mRNA transcript abundance also peaked during this developmental stage, paralleling the profiles of PATE enzyme activity and seed oil accumulation. A cotton *FatB* cDNA clone was isolated by screening a cDNA library with a heterologous *Arabidopsis FatB* probe (Pirtle et al., 1999, Plant and Cell Physiology 40: 155-163). The predicted amino acid sequence of the cotton PATE preprotein had 63% identity to the *Arabidopsis FatB* thioesterase sequence, suggesting that the cotton cDNA clone probably encoded a FatB-type thioesterase. When acyl-CoA synthetase-minus *E. coli* mutants expressed the cotton cDNA, an increase in 16:0 free fatty acid content was measured in the culture medium. In addition, acyl-ACP thioesterase activity assays in *E. coli* lysates revealed that there was a preference for palmitoyl-ACP over oleoyl-ACP *in vitro*, indicating that the cotton putative *FatB* cDNA encoded a functional thioesterase with a preference for saturated acyl-ACPs over unsaturated acyl-ACPs (FatA). Overexpression of the *FatB* cDNA in transgenic cotton resulted in elevated levels of palmitic acid in transgenic somatic embryos compared to control embryos. Expression of the anti-sense *FatB* cDNA in transgenic cotton plants

produced some plants with a dwarf phenotype. These plants had significantly smaller

mature leaves, all with smaller cells, suggesting that these plants may have less palmitic

acid available for incorporation into extraplastidial membrane lipids during cell

expansion. Thus manipulation of FatB expression in cotton directly influenced palmitic

acid levels. Collectively, data presented in this dissertation support the hypothesis that

there indeed is a palmitoyl-ACP thioesterase in cotton, encoded by the isolated *FatB*

cDNA, which plays a major role in regulating palmitic acid content of extraplastidial

complex glycerolipids. This work forms the basis for future studies of the influence of

palmitic acid content on plant membrane function and provides a key target for the

metabolic engineering of palmitic acid levels in storage oils of developing cottonseeds.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

iv

v

LIST OF TABLES

LIST OF FIGURES

.

Chapter 3

GENERAL INTRODUCTION

The major constituents of plant membranes and storage oils are glycerolipids

(Murphy, 1993). Acylglycerols, phospholipids, and glycolipids are the three major

classes of glycerolipids found in plants. Acylglycerols are the main component of seed

storage oils. These acylglycerols are usually in the form of triacylglycerols (TAGs),

which are made up of a single glycerol backbone to which three fatty acids are esterified.

The fatty acids found in storage lipids can range in length from 8 to 24 carbons (Murphy,

1993). The fatty acids found in membrane phospholipids of plants, which are important

components of all plant subcellular membranes, are usually 16 to 18 carbons in length.

These membrane lipids are made up of the glycerol backbone, with fatty acid groups

attached to the *sn*-1 and *sn*-2 positions and a phosphate ester group attached to the *sn*-3

position. The most common phosphate esters of plant phospholipids are choline,

glycerol, ethanolamine, serine, and inositol (Murphy, 1993). The third class of

glycerolipids, the glycolipids, is an important component of chloroplast membranes. In

particular, galactolipids account for 70-80% of the total lipids found in these membranes

(Murphy, 1993). Galactolipids consist of the glycerol backbone with fatty acid groups

esterified to the *sn*-1 and *sn*-2 positions and at least one galactose residue attached at the

sn-3 position.

The fatty acid groups of these glycerolipids are synthesized in the stromal compartment of plastids. Fatty acid biosynthesis is initiated by the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA (Browse and Somerville, 1991; Somerville et al., 2000). Two carbon units donated by malonyl-ACP are attached to the growing fatty acyl chain, which is esterified to an acyl-carrier protein (ACP). Monofunctional enzymes required for the extension of the acyl chains make up the type II fully dissociable fatty acid synthase (FAS) complex (Ohlrogge and Browse, 1995; Harwood, 1996; Kinney, 1997; Ohlrogge and Jaworski, 1997). Cleavage of the thioester bond by acyl-ACP thioesterases terminates acyl-chain elongation during fatty acid biosynthesis (Harwood, 1988; Harwood, 1996). Typically fatty acid chain elongation is terminated at C16:0 or C18:1 resulting in the production of 16:0-ACP and 18:1-ACP as the major products of plastid fatty acid biosynthesis. These fatty acid groups are then exported from the plastid to the endoplasmic reticulum (ER) for incorporation into glycerolipids of membranes or seed oils.

The mechanism for determining fatty acid chain length has been investigated extensively in the last decade (Browse and Somerville, 1991; Ohlrogge et al. 1993; Dörmann et al., 1994; Dörmann et al., 1995; Eccleston et al., 1996; Voelker, 1996; Somerville et al., 2000). These studies have indicated that the pools of fatty acids (as acyl-CoAs) available for export to extraplastidic compartments are determined by the substrate specificities of acyl-ACP thioesterases. Plant acyl-ACP thioesterases were initially characterized as enzymes, which were predominantly active towards oleoyl-ACP ($18:1^{\Delta 9}$-ACP) with lower activities toward other acyl-ACPs (Ohlrogge et al., 1978). Seed

specific medium-chain acyl-ACP thioesterases were later discovered when species that produced high levels of medium-chain fatty acids in their seeds were examined (Pollard et al., 1991; Voelker et al., 1992). When the DNA sequences from the oleoyl-ACP and medium-chain acyl-ACP thioesterases were compared, these sequences were found to fall into two groups. Two different types of thioesterases designated as FatA or FatB, with substrate preferences for unsatuarated acyl-ACPs or saturated acyl-ACPs, respectively, were originally classified according to Jones et al. (1995).

Recently an expressed sequenced tag (est) from *Arabidopsis* was found with a cDNA sequence that was similar to the medium-chain thioesterases (Dörmann et al., 1995; Dörmann et al, 2000). This was a surprising discovery since *Arabidopsis* plants do not accumulate medium-chain fatty acids in their tissues. Expression of the *AtFATB1* thioesterase in *Escherichia coli* revealed that this cDNA encoded an enzyme most active *in vitro* toward 16:0-ACP, with some overlapping activity for $18:1^{\Delta 9}$-ACP, 18:0-ACP, and 14:0-ACP (Dörmann et al, 1995). Unlike the previously discovered FatB thioesterases which were seed-specific, the AtFATB1 was found in other tissues such as leaves, roots, and siliques. The *Arabidopsis* cDNA clone encoded the first FatB long-chain acyl-ACP thioesterase, which was constitutively expressed. Transgenic *Arabidopsis* plants harboring the *AtFATB1* cDNA under the transcriptional control of a seed-specific promoter were produced and used to examine the *in planta* role of FatB thioesterases (Dörmann et al, 2000). Elevated levels of palmitic acid were detected in seeds overexpressing FatB. In addition, expression of the anti-sense *AtFATB1* cDNA under the transcriptional control of the Cauliflower Mosaic Virus 35S (CaMV 35S)

promoter resulted in a modest reduction of palmitic acid in seeds and flowers. Consequently Dörmann et al. (2000) concluded that the accumulation of palmitic acid in *Arabidopsis* was influenced by the activities of the AtFatB1 acyl-ACP thioesterase.

With the recent discovery of a constitutive *Arabidopsis* FatB thioesterase, Somerville et al. (2000) have proposed a new model for the compartmentation of lipid synthesis in plants. The current model suggests that some FatB isoforms are ubiquitously expressed while other FatB isoforms are expressed exclusively in seeds. The FatA $18:1^{\Delta 9}$-ACP thioesterases and the FatB 16:0-ACP thioesterases are proposed to be active in most tissues of all species, while other FatB isoforms are found only in seeds and are species-specific. The activities of the FatB seed-specific isoform results in the accumulation of medium-chain fatty acids in seed storage lipids (Voelker et al, 1992; Dörmann et al., 1993; Dehesh et al., 1996).

A cDNA clone presumably encoding a cotton palmitoyl-ACP thioesterase (FatB), which has 63% identity in the amino acid sequence to the *Arabidopsis* FatB long-chain acyl-ACP thioesterase, was recently isolated (isolated and sequenced by David Yoder and Mongkol Nampaisansuk in Dr. R. Pirtle's Laboratory; Pirtle et al., 1999). The current model proposed by Somerville et al. (2000) was developed based on work with A*rabidopsis* plants, and we chose to test this model in a crop plant system, specifically cotton. The goal in this dissertation research project was to characterize the cotton *FatB* cDNA clone isolated and to examine the *in planta* role of the palmitoyl-ACP thioesterase in cotton. Based on previous studies with acyl-ACP thioesterases, it is known that the free fatty acids released upon hydrolysis by these thioesterases are exported to the ER to

be further modified into membrane lipids or assembled into TAGs of seeds (Kinney, 1994; Harwood, 1996; Eccleston and Ohlrogge, 1998). In cottonseeds, the TAGs are comprised mostly of palmitic (16:0, 22%) and linoleic (18:2, 58%) acids, with considerably lower levels of oleic (18:1, 15%) and stearic (18:0, 2%) acids. By comparison, TAGs of other domestic oilseed crops such as canola, corn, and soybean, have considerably lower levels of palmitic acid (4%, 11%, 11%, respectively). We speculated that the relatively high levels of palmitic acid in cottonseeds may be due to the specific activity of a FatB palmitoyl-ACP thioesterase (PATE), which selectively prefers C16:0-ACPs as its substrates and influences the proportion of saturated fatty acids incorporated into extraplastidial lipids. We tested this hypothesis by (a) evaluating the expression of the endogenous FatB in different tissues / organs of cotton at different developmental stages and correlating relative transcript abundance with enzyme activity and palmitic acid content where appropriate (Chapter 1), by (b) expressing the cotton FatB in various *E. coli* strains to examine its functional activity *in vivo* and *in vitro* (Chapter 2), and by (c) manipulating the expression of this FatB in transgenic cotton to investigate its role in modulating palmitic acid content in cotton (Chapter 3).

In chapter one, I described the biochemical and molecular characterization of an endogenous acyl-ACP thioesterase in cotton. This preliminary work provided evidence for the existence of a FatB thioesterase in cotton, and suggested which tissues and at which developmental stage FatB was most active. Data from these experiments provided the basis for my hypothesis that a FatB palmitoyl-ACP thioesterase is involved in the regulation of palmitic acid content in cotton. In chapter two, I examined the functional

expression of the cotton *FatB* cDNA in transformed *E. coli* cells. To address if the isolated cotton *FatB* cDNA clone encoded a functional protein product, I conducted *in vitro* transcription/translation experiments and measured the recombinant FatB activity *in vivo* and *in vitro*. In chapter three, I examined the *in planta* role of the cotton FatB thioesterase by characterizing transgenic cotton plants harboring the PATE sense and anti-sense expression constructs under the transcriptional control of the Cauliflower Mosaic Virus (CaMV) 35S promoter. Transgenic plants were used as tools to elucidate the role of FatB in lipid accumulation. Specifically we explored the possibility that the manipulation of FatB expression would alter the palmitic acid content in cotton. These transgenic plants will provide the basis for future research to examine how palmitic acid levels influence plant growth and development.

The overall goal of this dissertation research was to gain a better understanding of the regulation of palmitic acid (16:0) synthesis and accumulation in cotton. Recent studies on transgenic *Arabidopsis* plants indicate that a FatB thioesterase is involved in the regulation of palmitic acid accumulation in some *Arabidopsis* tissues. Because the *Arabidopsis FatB* cDNA was used to isolate the cotton cDNA, we speculated that this cDNA was probably an *Arabidopsis* FatB homologue. However no biochemical or molecular evidence for an endogenous FatB acyl-ACP thioesterase in cotton has been reported and the overall role of FatB in plants still remains unclear. Although the role of FatB thioesterases has been examined in *Arabidopsis*, there are several beneficial reasons for further characterizing the FatB thioesterase specifically in cotton. Unlike *Arabidopsis*, cotton is an oilseed crop. FatB in cotton can be a potential metabolic target

for genetic engineering of cottonseed oil. Lipid biochemists are interested in altering

fatty acid compositions of seed storage oils, such that novel seed oils have lower

saturated fatty acids for health benefits. Alteration of FatB expression may influence

fatty acids available for membrane synthesis as well. The fatty acid types in membrane

lipids directly influence membrane fluidity, which can confer temperature-sensitivity.

Arabidopsis plants are cold tolerant, while cotton plants are cold sensitive. In addition,

Arabidopsis plants have different life cycles than cotton plants. *Arabidopsis* are annual

plants that complete one full life cycle in one growing season and can produce seeds,

which can withstand the winter season. However, cotton is a perennial plant that can

only grow in warm temperatures during the late spring and summer months. Perennial

plants usually die in the fall and emerge again in the following spring season. Thus the

investigation of the role of FatB thioesterases in the regulation of lipid metabolism should

provide insight into membrane function, and may have implications for engineering

temperature tolerance in cotton fibers and plants. Dörmann et al. (2000) investigated the

overexpression of FatB thioesterases only in seeds of *Arabidopsis*. Here we extended this

investigation by exploring the constitutive overexpression of FatB to examine the impact

of membrane function on plant growth and development. The following text describes

my results which are divided into three chapters: Chapter 1 – Biochemical and Molecular

Characterization of an Acyl-ACP Thioesterase in Cotton; Chapter 2 – Characterization

of the Cotton *FatB* cDNA in Transformed *E. coli* cells; Chapter 3 – Development and

Characterization of Transgenic Cotton Plants Harboring Sense and Anti-sense PATE

Expression Constructs.

CHAPTER 1

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF AN ACYL-ACP THIOESTERASE IN COTTON (*GOSSYPIUM HIRSUTUM* L.)

Abstract

The relatively high level of palmitic acid (22 mol%) in cottonseeds may be due in part to a palmitoyl-acyl carrier protein (ACP) thioesterase (PATE), which prefers C16:0-ACP as its substrate. In embryo extracts, PATE activity was highest at the maximum rate of reserve accumulation (oil and protein), occurring about 30-35 days post anthesis. Thioesterase activity toward oleoyl-ACP was relatively similar at all developmental stages examined, but was considerably lower than the PATE activity. In developing seeds and in cotyledons and hypocotyls of seedlings, the PATE activity predominated. A cotton PATE cDNA clone was isolated by screening a cDNA library with a heterologous *Arabidopsis FatB1* probe. The predicted amino acid sequence of the cotton PATE preprotein has a characteristic stromal-targeting domain and a 63% identity to the *Arabidopsis* long-chain

acyl ACP-thioesterase FatB1 sequence. Alkaline blot hybridization of cotton genomic

DNA with the *Arabidopsis FatB1* probe suggested the presence of at least two *FatB1*

thioesterase genes in cotton. Relative cotton *FatB1* transcript abundance was compared

by RT-PCR and slot blot hybridization analysis of total RNA extracts from embryos,

seedlings and leaves of mature plants. The cotton *FatB1* mRNA apparently was expressed

in all tissues but paralleled the profiles of PATE enzyme activity and seed oil

accumulation in embryos.

Abbreviations

ACP, acyl carrier protein; bp, base pairs(s); dpa, days post anthesis; GC, gas

chromatography; kb, kilobase(s) or 1,000 bp; nt, nucleotide(s); OTE, oleoyl-acyl carrier

protein thioesterase; PATE, palmitoyl-acyl carrier protein thioesterase; RT-PCR, reverse-

transcription-polymerase chain reaction; SSC, 150 mM sodium chloride and 15 mM

sodium citrate (pH 7.0); TAG(s), triacylglycerol(s); TLC, thin-layer chromatography.

Introduction

Higher plants synthesize fatty acids *de novo* in the stromal compartment of plastids with a type II, fully dissociable fatty acid synthase complex (Ohlrogge and Browse, 1995; Harwood, 1996; Kinney, 1997; Ohlrogge and Jaworski, 1997). Acyl chains esterified to an acyl carrier protein (ACP) undergo chain elongation by the sequential addition of two-carbon units from malonyl-ACP. Hydrolysis of the acyl-ACP thioester bond by an acyl-ACP thioesterase enzyme terminates acyl chain elongation. The plant acyl-ACP thioesterases are usually categorized by the preferences exhibited for their acyl-ACP substrates, specified by the degree of saturation and chain length of the acyl group (Voelker, 1996). Jones et al. (1995) originally classified the occurrence of two different types of thioesterases, designated as FatA and FatB, with substrate preferences for unsaturated or saturated acyl-ACPs , respectively.

These fatty acids synthesized in the plastids are exported for incorporation into complex glycerolipids of membrane or storage oil lipids (Figure 1). The fatty acid chains are cleaved from the ACPs by the acyl-ACP thioesterases and are exported to the endoplasmic reticulum (ER) as acyl-CoAs by an unknown mechanism. The fatty acid desaturases (Fad) in the ER modify the number of double bonds in the fatty acyl chains. Thus the acyl-ACP thioesterases and fatty acid desaturases together determine the types of fatty acids (fatty acyl chain length and degree of unsaturation, respectively), which are incorporated into membrane or oil glycerolipids.

FatB cDNAs encoding thioesterases have been characterized with substrate preferences for: short-chain C8-C10 acyl-ACPs, such as the *Cuphea palustris* FatB1 thioesterase (Dehesh et al., l996) or elm Fat B thioesterase (Voelker et al., 1997); for medium-chain C12-C14 acyl-ACPs, such as the California bay tree thioesterase (Voelker et al., 1992), the *Cuphea palustris* FatB2 thioesterase (Dehesh et al., l996), or the nutmeg FatB thioesterase (Voelker et al., 1997); and for long-chain C16-C18 acyl-ACPs, such as the *Arabidopsis* FatB1 thioesterase (Dörmann et al., 1995) or the *Cuphea hookeriana* Fat B thioesterase (Jones et al., 1995). Consequently, the complement of thioesterase enzymes expressed in developing oilseeds determines to a great extent the available pool of fatty acids that can be incorporated into TAGs (Figure 1). Efforts in altering seed oil profiles have been successful by altering the expression of acyl-ACP thioesterases, and have led to the commercial production of several modified seed oils, such as high-laurate canola and high-oleate soybean oils (reviewed by Voelker, 1996; DelVecchio, 1996; Kinney, 1997).

One long-term goal is to alter the fatty acid composition of cottonseed oil. In this chapter, I described the biochemical and molecular characterization of an endogenous FatB palmitoyl-ACP thioesterase in cotton. Cotyledons of cotton were the physiological/developmental model used to examine the regulation of lipid metabolism in embryos, germinated dark-grown seedlings, and green cotyledons of seedlings exposed to light (Figure 6). Cotyledons of cotton embryos are involved in lipid accumulation for storage. The synthesis of TAGs is highest at this developmental stage. Cotyledons of germinated cotton seedlings are involved in lipid mobilization for seed germination and

seedling growth. These same organs differentiate to leaf-like structures of green

seedlings upon exposure to light and begin to synthesize chemical energy from light

energy by photosynthesis (Trelease and Doman, 1984). At this developmental stage,

lipids are incorporated into membranes for leaf expansion. This model system was used

to survey endogenous thioesterase activity and relative transcript abundance and to

correlate thioesterase expression with a potential role for palmitoyl-ACP thioesterases in

cotton. Altogether, the results presented in this chapter provide evidence that a FatB

acyl-ACP thioesterase activity is present in cotton and may be responsible for the high

level of palmitic acid incorporated into cottonseed oil.
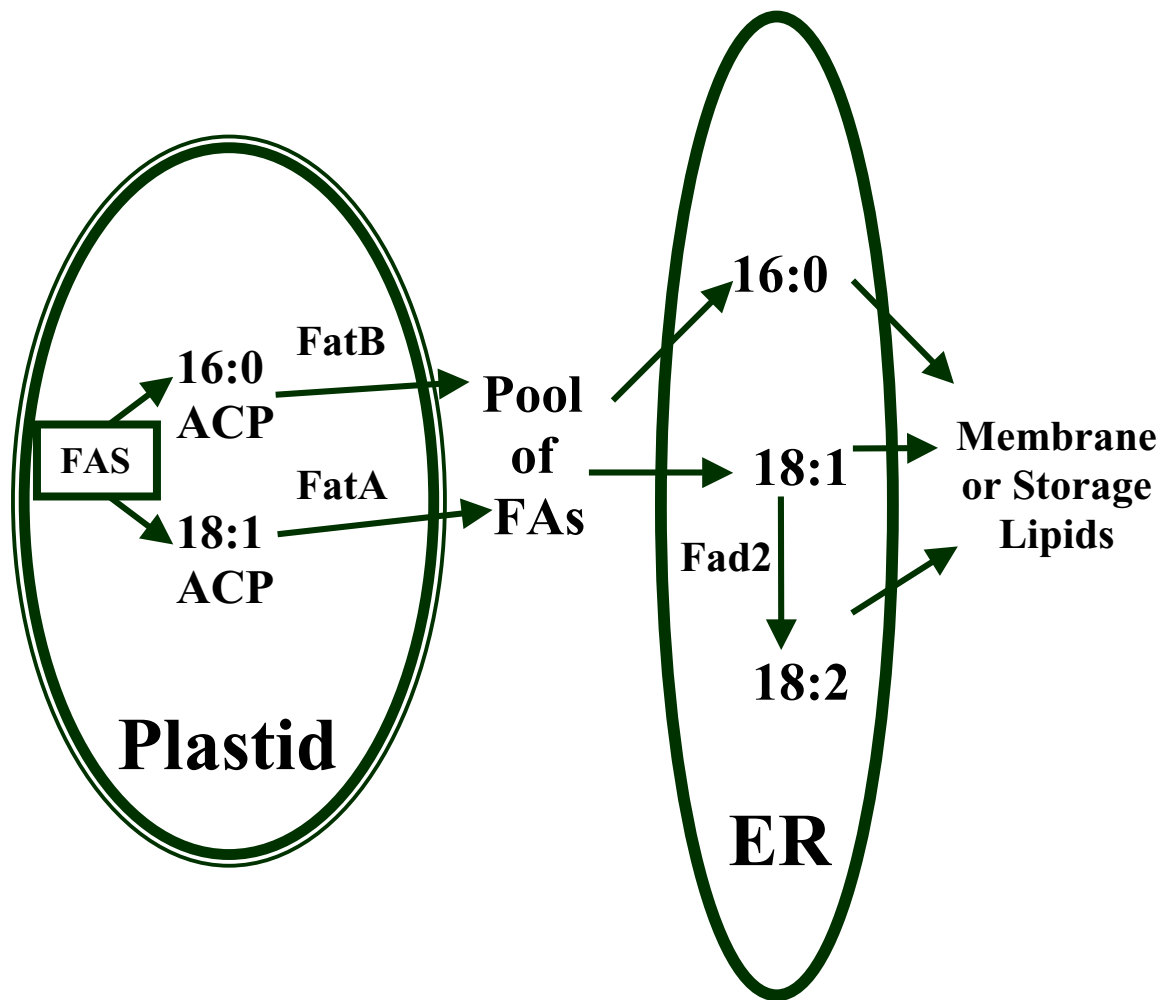
Figure 1. Simplified scheme for the cellular compartmentation of membrane lipids and/or seed oil synthesis. Higher plants synthesize fatty acids *de novo* in the stromal compartment of plastids. Acyl chains esterified to an acyl carrier protein (ACP) undergo chain elongation by the sequential addition of two carbon units, donated by malonyl-ACP. Hydrolysis of the acyl-ACP thioester bond by an acyl-ACP thioesterase terminates

acyl chain elongation. The current model accounts for two different types of

thioesterases in plant plastids, which are categorized by the preference exhibited for their

acyl-ACP substrates. They are designated as FatA and FatB thioesterases, with substrate

preferences for unsaturated or saturated acyl-ACPs, respectively. Thus, acyl-ACP

thioesterases determine the pool of available fatty acids exported from seed plastids for

plant membrane or storage oil (mostly as triacylglycerols, TAGs) assembly. Fatty acid

desaturases (Fad), located in the endoplasmic reticulum (ER), then modify the number of

double bonds in the fatty acyl chains which are incorporated into complex glycerolipids

(Adapted from Somerville et al., 2000).

Materials and Methods

Plant Material

Cotton (*Gossypium hirsutum* L., cv. Paymaster HS26) plants were grown in the greenhouse under conditions previously described (Chapman and Sprinkle, 1996). Developing bolls were collected at specific days post anthesis (20 dpa - 50 dpa) from plants with flowers tagged at anthesis. The embryos were excised from ovules and frozen in liquid nitrogen and stored at -80°C for RNA, protein, and lipid extractions and analyses. Cotyledons, hypocotyls, and roots were collected from seedlings germinated at 30°C and grown in the dark for 48 h. Young leaves of mature plants were harvested immediately before RNA or DNA isolation. Seeds were a gift from Dr. John J. Burke (USDA-ARS, Lubbock).

Southern Blot Analysis

Cotton genomic DNA was isolated from nuclei of young leaves according to Paterson et al. (1993). The DNA (15 μg) was digested with 10 units of *Eco*RI or *Hind*III (Promega) for several hours at 37°C. The genomic restriction fragments were resolved on 0.8% agarose gels and transferred by alkaline blotting (Reed and Mann, 1985) to Zeta-probe nylon membrane (BioRad). Blots were hybridized at 60°C overnight in 5X SSC, 5% dextran sulfate, 0.5% SDS, denatured sheared salmon sperm DNA (100 μg/mL), and nonradioactive DNA probe generated from the 1.4-kb *Arabidopsis* TE 3-2 SalI/*Eco*RV fragment (portion of *Arabidopsis FatB* cDNA) by random priming using the Gene Images system from Amersham. After a final rinse with 1X SSC, the hybridizing

fragments were visualized by chemiluminescence according to the manufacturer's

protocol (Gene Images CDP-Star detection module, Amersham).

Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and Slot Blot

Hybridization

Total RNA was isolated from developing embryos, different organs of 2-d old

seedlings (cotyledon, hypocotyl, and root), and mature leaves using a modified, hot

borate procedure described by Wan and Wilkins (1994). The RNA yield and quality

were evaluated spectrophotometrically and by analytical gel electrophoresis on a 1%

agarose gel by the procedure of Wilkins and Smart (1996). RNA concentrations were

determined from absorbance readings and were adjusted accordingly, based on the

relative intensities of the 18S and 28S rRNAs in each sample. Equivalent amounts of

total RNA were used as templates with the Promega RT-PCR Access kit. Reverse

transcription of total RNA (approximately 200 ng) from various organs of cotton plants

and from embryos at different development stages were conducted in a Perkin Elmer

2400 thermal cycler. RT-PCR conditions were as follows: First-strand cDNA synthesis

was carried out at 48°C for 45 min. Subsequent amplification of a targeted region within

the PATE cDNA coding region was achieved through 35 cycles of 94°C for 30 sec, 56°C

for 1 min, and 68°C for 2 min followed by a final polymerization step at 68°C for 7 min.

The PATE cDNA primers from Biosynthesis, Inc. for PCR amplification were 5'-

GGGTGATGTTGTTCAAGTCG-3' (forward) and 5'-cotton ATCCAGCC-3' (reverse),

and used to amplify the appropriate fragment of 354 bp (shown by arrows in Figure 2).

Actin primers (5'-TGCAGGTCGTGATCTAACCG-3' (forward) and 5'-

CCTTGGAAATCCACATCTGC-3' (reverse)) were selected from the partial actin cDNA sequence (GenBank Accession D88414), and used to amplify a fragment of 539 bp. The PCR products were separated on 3% agarose gels and the bands visualized following ethidium bromide staining.

For RNA slot blot hybridization (conducted in collaboration with Dr. R. Pirtle's laboratory), 10 µg of total RNA for each of the various tissues and developmental stages was denatured in formaldehyde prior to immobilization on a positively-charged membrane (Hybond-N$^+$, Amersham) in a slot-blot manifold (Bio-Rad Bio-Dot SF) by the procedure described by Sagerström and Sive (l996). The slot- blot membranes were prehybridized for 3 h at 60°C in a solution containing 6X SSC, 5X Denhardt's solution, 0.5% SDS, and denatured salmon sperm DNA (100 µg/mL). Hybridization was done overnight at 60°C in a solution containing 6X SSC, 5X Denhardt's solution, 0.5% SDS, 2 mM EDTA, 2.5 mM Na pyrophosphate (pH 8.0), 20 mM Tris-HCl (pH 8.0), denatured salmon sperm DNA (100 µg/mL) and ^{32}P-labeled PATE or actin probe. The homologous PATE DNA fragments were derived from the cotton PATE cDNA and radiolabeled with ^{32}P by a random priming procedure (Feinberg and Vogelstein, 1983). The homologous actin DNA fragments were generated by random priming using the 539-bp actin RT-PCR fragment described above as template. Following exposure of the X-ray film, the appropriate sections of the slot-blot hybridization membranes were excised and radioactivity was quantified in a liquid scintillation counter (Beckman LS Model 3801).

DNA Sequencing

RT-PCR amplification products were fractionated on 1.5% agarose (PE-XPRESS, Perkin-Elmer) gels in standard TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) for 2 h at 70V. Fragments of predicted sizes were excised from the gel and purified using either a Prep-a-Gene (BioRad) DNA purification kit. Purified DNA was ligated into *Eco*RV-digested pZErO-2™ plasmids (Invitrogen) with a Fast-Link DNA ligation kit (Epicentre Technologies). *E. coli* cells (Top 10 ™, Invitrogen), made competent with 0.1 M calcium chloride and 0.01 M rubidium chloride (Seidman et al., 1997), were transformed with the pZErO-2™ plasmid vectors and plated on selective agar, containing NZY media (21 mg/mL), 0.05 mg/mL kanamycin, and 1 mM IPTG. These plasmids harbor the lethal gene *ccd*B (control of cell death, induced by the *lac* Z promoter) and kanamycin resistance. Only the cells, which carry plasmids with foreign DNA (the RT-PCR product inserts disrupt the *ccd*B gene), will be viable on these plates. Plasmid DNA was purified (Wizard Plus SV miniprep DNA purification kit, Promega) from 10 mL cultures (NZY, 21 mg/mL; 0.05 mg/mL kanamycin) inoculated with single positive colonies and grown to log phase. The plasmid DNA (about 1 μg) was digested with *Xba*I and *Sac*I to verify RT-PCR inserts. DNA purity and quantity were estimated spectrophotometrically.

Both strands of the 354 bp RT-PCR product were sequenced and compared to the cDNA sequence (Pirtle et al., 1999). RT-PCR fragments subcloned into double-stranded pZErO-2™ plasmid vectors were sequenced with IRD41-labeled M13 forward and

reverse primers (LI-COR). The sequences (dideoxy-termination reactions) were verified

completely (Epicentre SequiTherm EXCEL II Long-Read DNA Sequencing Kit-LC

fluorescent-labeled primer sequencing kit with 7-deaza-dGTP) on a LiCOR 4000L semi-

automated sequencer (sequenced in Dr. J. Knesek's Laboratory) according to

manufacturer's protocols.

Preparation of Cell Free Homogenates

The various cotton tissues were chopped manually with a single edge razor blade

before homogenization. The tissues were homogenized in 1:1 (w/v) of 400 mM sucrose,

100 mM $NaPO_4$ (pH 7.2), 10 mM KCl, 1 mM EDTA, and 1 mM $MgCl_2$, in a glass vessel

with a motorized teflon pestle. The homogenates were filtered through four layers of

cheese cloth and the filtrates were immediately assayed for thioesterase activity.

Preparation of Substrates

Acyl-ACPs were synthesized according to Post-Beittenmiller et al. (1991). The

50 μL reaction mixtures for acyl-ACP synthesis contained 400 mM Tris (pH 8.0), 40

mM $MgCl_2$, 1.6 M $LiCl_2$, 100 mM ATP, 100 mM DTT, 0.3 mg/mL *E. coli* ACP (Sigma),

60 μM [^{14}C]-fatty acid (either [1-^{14}C]-palmitic acid (56.0 mCi/mmol) or [1-^{14}C]-oleic

acid (50 mCi/mmol) from Dupont/ NEN), and 4 units of *E. coli* acyl-ACP synthetase

(Sigma). The reaction mixtures were incubated at 37°C for 3-6 h with gentle shaking (60

rpm). The reactions were stopped by a tenfold dilution into 50 mM MES, pH 6.1. The

acyl-ACPs were purified by bulk adsorption on DEAE-cellulose (Whatman DE52)

equilibrated in 50 mM MES, pH 6.1 (0.5 mL DEAE-cellulose slurry per 0.5 mL reaction

mixture). The mixtures were centrifuged in a microcentrifuge at 16,000 x*g* for 10 min.

The supernatants were decanted and the pellets washed twice with 5-10 mL of a solution of 80% isopropanol, 20% 10 mMKPO$_4$, pH 6.0 and 100 mM NaCl and once with 50 mM MES, pH 6.1. The acyl-ACPs were eluted from the DEAE-cellulose with 100 μL of 0.4 M LiCl and 50 mM MES, pH 6.1. The mixtures were centrifuged as before and the supernatants with the acyl-ACPs were transferred into fresh microcentrifuge tubes and stored at -20°C.

Thioesterase Assays

Enzyme reactions were started by adding the [^{14}C]acyl-ACP to the cellular homogenates.   The mixtures were incubated at 37°C with gentle shaking (60 rpm), and the reactions were terminated after 30 min with the addition of an equal volume of isopropanol containing 1 mM oleic acid. The free fatty acids were extracted with petroleum ether saturated with 50% isopropanol. These samples were dried under nitrogen and then resuspended in 50 μL of chloroform.   Radioactivity in half of the sample was quantified by counting in a Beckman LS Model 3801 Liquid Scintillation Counter. The remainder was analyzed by TLC and radiometric scanning (BioScan System 200) to confirm the release of [1-^{14}C]-palmitic acid or [1-^{14}C]-oleic acid.

 Lipid Analysis

Non-polar lipids were fractionated on TLC plates (Whatman silica gel G-60, 250 μm) for 45 min in hexane:diethylether (80/20, v/v), and the relative amounts of triacylglycerols and free fatty acids in each sample were calculated by scanning densitometry (NIH Image, v. 6.1) in comparison with triolein and oleic acid standards (Chapman and Sprinkle, 1996). Free fatty acids were extracted from appropriate regions

of separate TLC plates and transesterified (Christie, 1982) for separation and

quantification by gas chromatography on a Hewlett-Packard 5890 Series II Gas

Chromatograph using conditions previously described (Chapman and Trelease, 1991).

Results

Isolation and DNA sequence analysis of a cotton palmitoyl- ACP thioesterase (*FatB*) cDNA clone

The cDNA insert in the pBluescript SK(-) phagemid derivative designated

pSKCPc115 (clone isolated and sequenced by David Yoder and Mongkol Nampaisansuk

in Dr. R. Pirtle's Laboratory) was determined to be 1,694 bp, and the deduced amino

acid sequence is shown with the nucleotide sequence in Figure 2. The cDNA sequence

corresponded to the DNA sequence of a cotton genomic clone encompassing this PATE

gene (Yoder et al., 1999) and shared considerable similarity at the amino acid sequence

level with other acyl-ACP thioesterases (shown in Figure 3). The 3'-untranslated region

of this cDNA clone is 461 bp long, with the likely poly (A) polymerase near-upstream

(AATAAA-like) element (Hunt, 1994), being 5'-AATGAA-3' (nt 1685 to 1690 in Figure

2), 19 bp upstream from the poly(A) cleavage/polyadenylation site at nt 1703. The

presumptive cotton PATE preprotein is 413 amino acids in length, with typical stroma-

targeting domains in the N-terminal region (Cline and Henry, 1996), denoted by

underlining in Figure 2.

Alignment of deduced amino acid sequences (completed by David Yoder in Dr.

R. Pirtle's Laboratory) for the plant acyl-ACP thioesterases in Figure 3 indicates that

there is a 63% identity between the cotton and *Arabidopsis* preproteins, a 54% identity

between the cotton and *Cuphea hookeriana* preproteins, whereas there is only a 22%

identity between the cotton and *Brassica* preproteins. Similar identities of about 60%

occur between the predicted sequences among members of either the FatA or FatB types,

whereas identities between members of the two groups are typically much less, being

around 30% (Voelker, l996). Based upon this sequence similarity, we concluded that this

cotton cDNA likely encode a member of the FatB class of thioesterases.

Genomic Blot Hybridization Analysis

Analysis of cotton genomic DNA by alkaline blot hybridization (shown in Figure

4) revealed four hybridizing fragments following cleavage with either *Hin*dIII or with

*Eco*RI. The sizes of the *Hin*dIII fragments were deduced to be 4.2 kb, 3.1 kb, 2.2 kb,

and 1.6 kb, while the *Eco*RI fragments were determined to be 10.0 kb, 7.6 kb, 5.4 kb, and

4.0 kb. Based on the genomic *FatB* sequence (Yoder et al., 1999), we predicted that the

Arabidopsis probe should hybridize to one *Hin*dIII fragment and two *Eco*RI fragments.

Since there are four different hybridizing genomic fragments under relatively stringent

hybridization conditions (1XSSC, 60°C) for two different restriction endonucleases, it is

likely that there are more than one *PATE* genes in the allotetraploid cotton genome.

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  1 (M   V   A)  T   A   V   T   S   A   F   F   P   V   T   S   S   P   D   S   S
    (ATGGTTGCT)ACTGCTGTGACATCGGCGTTTTTCCCAGTCACTTCTTCACCTGACTCCTCT  +60
 21  D   S   K   N   K   K   L   G   S   I   K   S   K   P   S   V   S   S   G   S
    GACTCGAAAAACAAGAAGCTCGGAAGCATCAAGTCGAAGCCATCGGTTTCTTCTGGAAGT  +120
 41  L   Q   V   K   A   N   A   Q   A   P   P   K   I   N   G   T   V   A   S   T
    TTGCAAGTCAAGGCAAATGCTCAAGCACCTCCGAAAATAAACGGCACTGTGGCGTCGACG  +180
 61  T   P   V   E   G   S   K   N   D   D   G   A   S   S   P   P   P   R   T   F
    ACTCCCGTGGAAGGTTCCAAGAACGATGACGGTGCAAGTTCCCCTCCTCCTAGGACGTTT  +240
 81  I   N   Q   L   P   D   W   S   M   L   L   A   A   I   T   T   I   F   L   A
    ATCAACCAGTTACCTGATTGGAGCATGCTTCTTGCTGCTATCACAACCATTTTCTTGGCT  +300
101  A   E   K   Q   W   M   M   L   D   W   K   P   R   R   P   D   M   V   I   D
    GCTGAGAAGCAGTGGATGATGCTTGATTGGAAGCCGAGGCGGCCTGACATGGTCATTGAT  +360
121  P   F   G   I   G   K   I   V   Q   D   G   L   V   F   S   Q   N   F   S   I
    CCGTTTGGCATAGGGAAGATTGTTCAGGATGGTCTTGTTTTCAGTCAGAACTTCTCGATT  +420
141  R   S   Y   E   I   G   A   D   Q   T   A   S   I   E   T   L   M   N   H   L
    AGATCATATGAGATAGGCGCTGATCAAACAGCATCCATAGAGACACTAATGAATCATTTA  +480
161  Q   E   T   A   I   N   H   C   R   S   A   G   L   L   G   E   G   F   G   A
    CAGGAAACAGCTATAAATCATTGTCGAAGTGCTGGACTGCTTGGAGAAGGTTTTGGTGCA  +540
181  T   P   E   M   C   K   K   N   L   I   W   V   V   T   R   M   Q   V   V   V
    ACACCTGAGATGTGCAAGAAGAACCTAATATGGGTTGTCACACGGATGCAAGTTGTGGTT  +600
201  D   R   Y   P   T   W   G   D   V   V   Q   V   D   T   W   V   S   A   S   G
    GATCGCTATCCTACTTGGGGTGATGTTGTTCAAGTCGACACTTGGGTCAGTGCATCGGGG  +660
221  K   N   G   M   R   R   D   W   L   V   S   N   S   E   T   G   E   I   L   T
    AAGAATGGCATGCGAAGAGATTGGCTTGTCAGCAATAGTGAAACTGGTGAAATTTTAACA  +720
241  R   A   T   S   V   W   V   M   M   N   K   L   T   R   R   L   S   K   I   P
    CGAGCCACAAGTGTATGGGTGATGATGAATAAACTGACTAGAAGGTTATCTAAAATCCCA  +780
261  E   E   V   R   G   E   I   E   P   F   F   M   N   S   D   P   V   L   A   E
    GAAGAGGTTCGAGGGGAAATAGAACCTTTTTTTATGAATTCAGATCCTGTTCTGGCTGAG  +840
281  D   S   Q   K   L   V   K   L   D   D   S   T   A   E   H   V   C   K   G   L
    GATAGCCAGAAACTAGTGAAACTCGATGACAGCACAGCTGAACACGTGTGCAAAGGTTTA  +900
301  T   P   K   W   S   D   L   D   V   N   Q   H   V   N   N   V   K   Y   I   G
    ACTCCTAAATGGAGCGACTTGGATGTCAACCAGCATGTCAATAATGTGAAGTACATTGGC  +960
321  W   I   L   E   S   A   P   L   P   I   L   E   S   H   E   L   S   A   L   T
    TGGATCCTTGAGAGTGCTCCATTACCAATCTTGGAGAGTCACGAGCTTTCCGCCTTGACT +1020
341  L   E   Y   R   R   E   C   G   R   D   S   V   L   Q   S   L   T   T   V   S
    CTGGAATATAGGAGGGAGTGCGGGAGGGACAGCGTGCTGCAGTCACTGACCACTGTGTCT +1080
361  D   S   N   T   E   N   A   V   N   V   G   E   F   N   C   Q   H   L   L   R
    GATTCCAATACGGAAAATGCAGTAAATGTTGGTGAATTTAATTGCCAACATTTGCTCCGA +1140
381  L   D   D   G   A   E   I   V   R   G   R   T   R   W   R   P   K   H   A   K
    CTCGACGATGGAGCTGAGATTGTGAGAGGCAGGACCCGATGGAGGCCTAAACATGCCAAA +1200
401  S   S   A   N   M   D   Q   I   T   A   K   R   A   TER
    AGTTCCGCTAACATGGATCAAATTACCGCAAAAAGGGCATAGAAATCCAAGTAATCTCAT +1260
    TGCTGTGTGTAGTATCTATCGTGCTCTTTTCGGATTTATATACATATATTCCTTATGATT +1320
    ATTAGTCTTCCTTTGAGAAAAAAAAAGGGGGTTGTAATTAGGCTTGTTTAGGAGTCGGGT +1380
    TTTCGTACATAGCCTTGTAAGGCTCAGCTCGTATGACCCGAGCCTCGGACACGGATTTTG +1440
    TGAAGTTGGGCCCGTGCCCTAACCAGCATAGGCTCTTTCCATGGAAAGGTGGGTCTGCTT +1500
    TTGAAAAATTGAATAGCCATGTGAGATGGCTCTCTCCCTACATTATGGGCTTTTAACCAG +1560
    TTAGAGACCGGGTAGTTTAGGATAAAATTTATCTTTAATTTGGGAGGATTTGTATATTTT +1620
    TTTGCCTTTATTTTAACCTAAATTTGCTTATAATTATTTGGTTTTATATTTAGGTATTG  +1680
    AATCAATGAAGTTTTTAAATTTT                                       +1703
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Figure 2. The nucleotide and deduced amino acid sequences for the cotton *PATE* cDNA

clone SKCPc115b (isolated and sequenced by David Yoder and Mongkol Nampaisansuk

in Dr. R. Pirtle's Laboratory). The nucleotide sequence has been assigned the GenBank

23

Accession Number AF034266. The numbering on the right refers to nucleotide residues,

whereas the numbering on the left denotes amino acid residues. As described in the text,

the first nine nucleotides (shown in parentheses), corresponding to the three amino acids

MVA at the N-terminus of the PATE preprotein were determined from the DNA

sequence of the corresponding region of the PATE gene (unpublished results). The

sequence corresponding to the presumptive poly (A) polymerase near-upstream element

(nt 1685 to 1690) is underlined.   The stromal-targeting domains (Cline and Henry, 1996)

of the PATE preprotein corresponding to the N-terminal region (MVATAVTSAFF,

amino acids 1-11), the variable middle region (TSSPDSSDSKNKKLGSIKSKPSVSSGS,

residues 14-40), and the carboxy-proximal region (VKANA, residues 43-47) are denoted

by underlining the respective amino acid domains. The putative transient peptide

cleavage site is probably L84, when compared with other thioesterase preprotein

sequences (Gavel and von Heijne, 1990; Dörmann et al., 1995; Cline and Henry, 1996).

The catalytic cysteine required for formation of the covalent thiol enzyme intermediate

and the histidine involved in general base catalysis in the plant acyl-ACP thioesterases

(Yuan et al., 1996) would most likely correspond to the homologous residues C347 and

H312 in the cotton amino acid PATE sequence. The two other conserved histidine

residues (Yuan et al., 1996) would correspond to the homologous H167 and H377

residues above. The sequences used for designing the forward and reverse primers for

RT-PCR are indicated by arrows.

Figure 3. Alignment of the predicted amino sequences of plant acyl-ACP thioesterase

preproteins (Completed by David Yoder in Dr. R. Pirtle's Laboratory). The amino acid

sequences were aligned using the DNASIS software from Hitachi. The thioesterase sequences compared are: *Gossypium hirsutum* FatB1 thioesterase (GHRFATB1, Genbank Accession number AF034266), *Arabidopsis thaliana* FatB1 thioesterase (ARABPATE, Genbank Accession number Z36911), *Cuphea hookeriana* FatB1 thioesterase (CUHFATB1, Genbank Accession number U17076), *Cuphea palustris* FatB2 thioesterase (CPALFTB2, Genbank Accession number X76561), and *Brassica rapa* FatA1 thioesterase (BRAPFTA1, Genbank Accession number U17098). Completely conserved amino acid residues are indicated by reverse contrast.
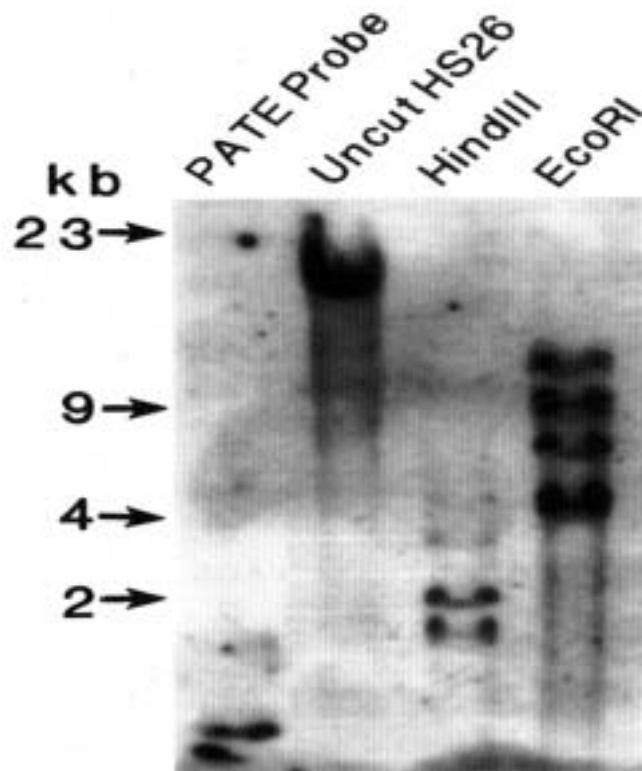
Figure 4.   Genomic blot hybridization of cotton DNA (cv Paymaster HS26). The cotton

DNA was digested with *Hind*III or *Eco*RI. The digestions were stopped by addition of 5

μL of a 5X gel loading buffer consisting of 50mM Na$_2$EDTA (pH 8.0), 25% glycerol,

0.5% SDS, 0.1% bromophenol blue, and 0.1% xylene cyanol and incubation at 70°C for 5

min. Undigested (uncut) DNA was also electrophoresed. The nylon membrane replica

was hybridized overnight (60°C) using a heterologous probe generated from a 1.4-kb

SalI/*Eco*RV fragment from the *Arabidopsis* cDNA clone TE 3-2. The membrane was

washed under moderately stringent conditions at 60°C in 1X SSC for 30 min. The

heterologous probe was nonradioactively labeled by random priming (Gene Images kit,

Amersham) and visualized by chemiluminescent staining the alkaline phosphatase

activity. The sizes (in kb) of standard DNA fragments obtained by digesting λ DNA

with *Hind*III are shown at the left.
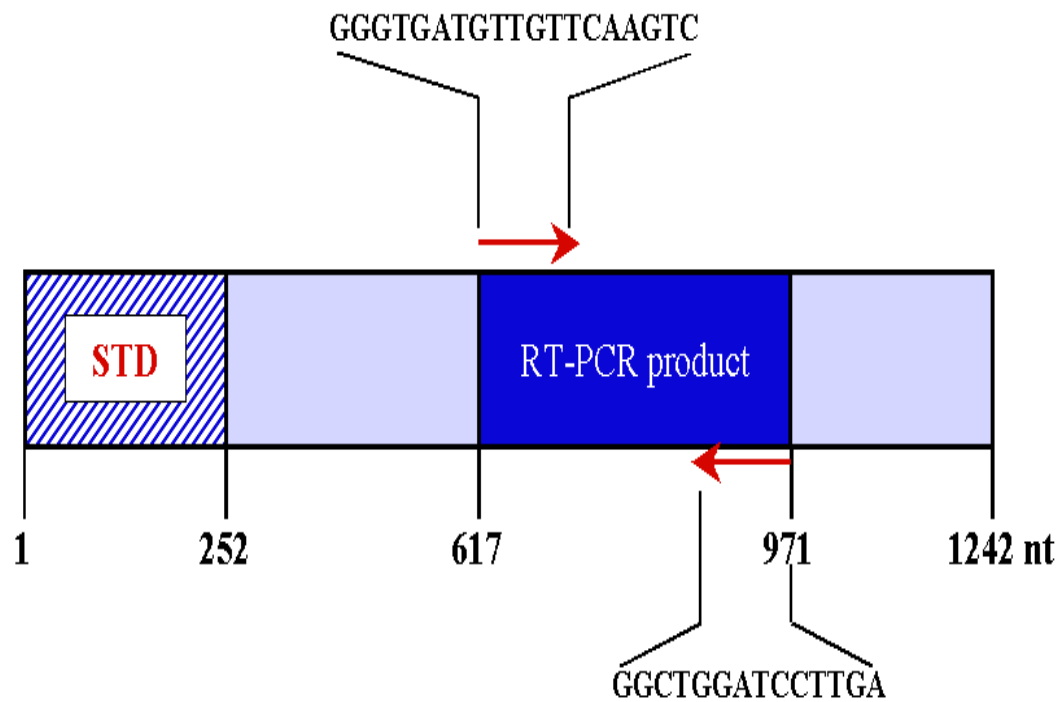
Figure 5. Schematic diagram illustrating the RT-PCR primers used to amplify a central region of the cDNA clone, resulting in a 354 bp DNA product (as depicted in Figures 6 and 7).

Tissue-Specific Expression and Developmental Studies

The palmitoyl-ACP and oleoyl-ACP thioesterase specific activities were compared in cell-free extracts of developing embryos, cotyledons, hypocotyls, roots of 2-d old seedlings, and leaves of mature plants (Figure 6, Figure 7A). The highest thioesterase activity was observed in homogenates of 30 dpa embryos, while the lowest activity was detected in homogenates of mature leaves. Furthermore, activity toward palmitoyl-ACP predominated over activity toward oleoyl-ACP in embryos, cotyledons, and hypocotyls, while the activities toward palmitoyl-ACP and oleoyl-ACP were comparable in seedling roots and mature leaves. The relative expression of cotton PATE determined by RT-PCR (Figure 7B) indicates that PATE mRNA is present in all tissues, including 30 dpa embryos (E), cotyledons (C), hypocotyls (H), roots (R), and mature leaves (L). Although not quantitative, the 354 bp PATE RT-PCR product appears to be more abundant in embryos than in the other tissues examined. Both strands of this 354 bp cotton PATE RT-PCR fragment generated were sequenced to confirm that the fragment was indeed amplified from PATE mRNA. The 539 bp actin RT-PCR product serves as a positive control for the constitutive expression of a housekeeping mRNA in total RNA extracts. Despite the double band observed in the actin embryo (E) lane, there seems to be relatively equal amounts of total RNA template in each RT-PCR reaction.

Figure 6: Cotton tissues and organs analyzed in thioesterase activity assays and expression studies. The upper left photo is of 30 dpa embryos. This is the stage of maximum storage lipid accumulation. The upper right photo is of 2-d old germinated seedlings (a pair of cotyledons at the top, hypocotyls as the central stem-like organ, connected to roots of the seedlings). This is the stage of lipid mobilization. The bottom photo is of cotton seedlings active in photosynthesis. Mature leaves (a cluster of four leaves in this photo) emerge from the apical meristem. The lower two green leaf-like organs are the cotyledons of the seedlings which undergo a developmental transition at the physiological and cellular levels to switch from heterotrophic to photoautotrophic growth.
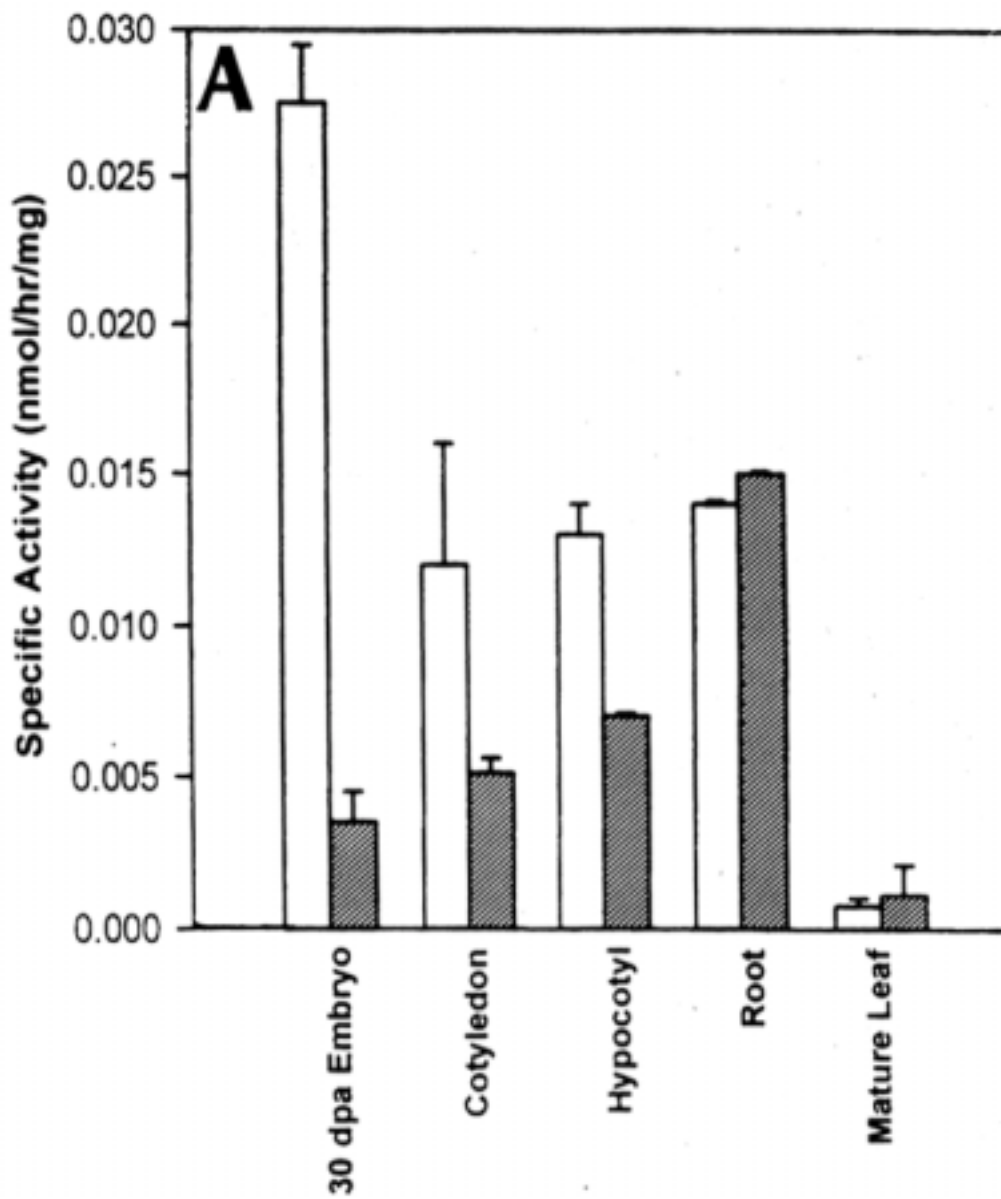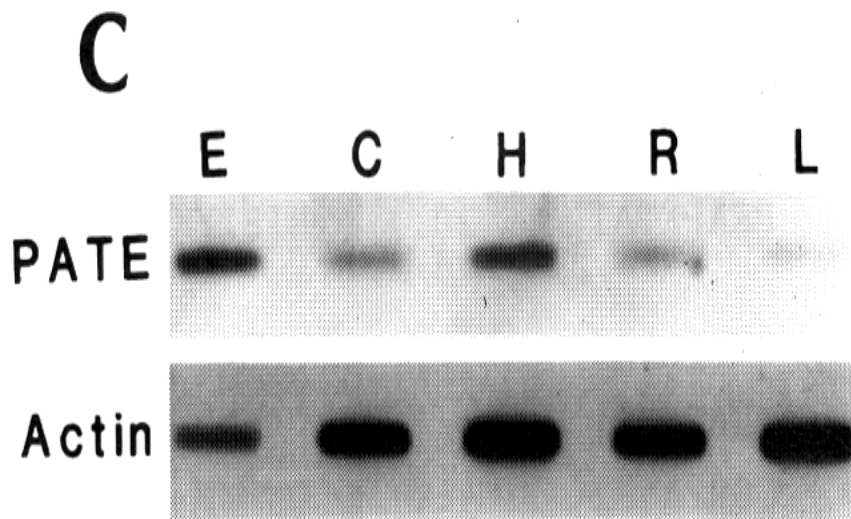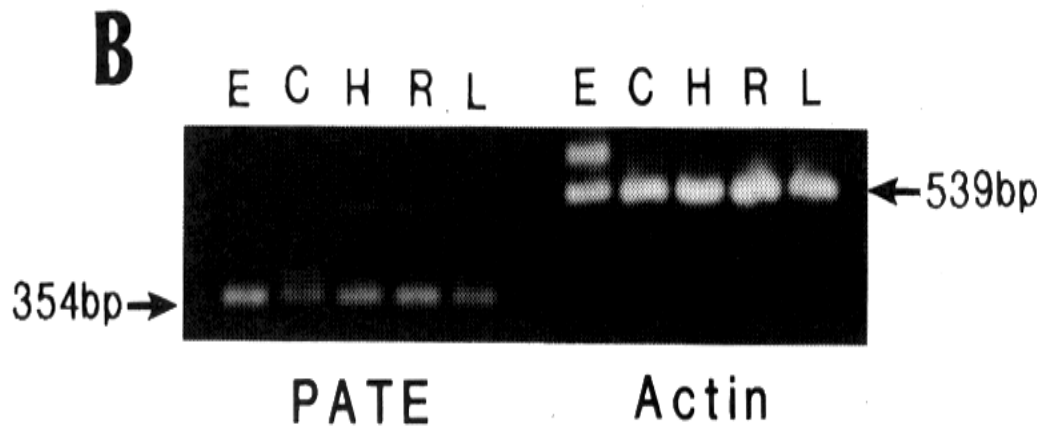
Figure 7. Thioesterase activity and relative transcript abundance in cell-free homogenates from cotton embryos, different organs of 2-d old seedlings (cotyledons, hypocotyls and roots), and leaves of mature plants. (A) Thioesterase activity was measured toward [^{14}C]-palmitoyl-ACP (open bars) or [^{14}C]-oleoyl-ACP (cross-hatched

bars) synthesized and purified as described in Methods. The values shown represent the mean and range from three independent experiments. (B) The relative transcript abundance was evaluated by RT-PCR with 0.2 µg total cotton RNA in each reaction for each tissue (30 dpa embryo, E; cotyledon, C; hypocotyl, H; root, R; and leaf, L) using the cotton PATE forward and reverse PCR primers (Figure 5) and the cotton actin forward and reverse PCR primers. The major RT-PCR products of the predicted size for the PATE product of 354 bp and actin product of 539 bp were detected by ethidium bromide staining following electrophoresis in 3% agarose gels. (C) Slot-blot hydridization of 10 µg total cotton RNA in each reaction for each tissue (as in B above) with homologous ^{32}P-labeled cotton PATE and actin probes generated by random priming. (Slot blots were conducted in Dr. Pirtle's Laboratory with the assistance of Irma Pirtle.)

As shown in Figure 7C, a similar pattern of expression was obtained with slot blots of total RNA probed with random-primed fragments derived from cotton PATE cDNA and the cotton actin RT-PCR fragment, with the PATE mRNA apparently being the most abundant in embryos (^{32}P dpm ratio of embryo PATE/actin of 1.1 relative to an average ratio of 0.23 for the others).

As shown in Figure 8A, the developmental profile of PATE specific activity (solid circles) indicates that thioesterase activity was highest at the stage of maximum oil (solid triangle) and protein (open squares) reserve accumulation. Free fatty acid content (open circles) in the embryo extracts also peaked during this stage. GC analysis of fatty acid methyl esters confirmed that the predominant free fatty acid was mostly palmitic acid (data not shown). Oleoyl-ACP thioesterase activity (solid squares) was considerably lower than PATE activity in embryo extracts and was similar at all developmental stages. A comparable profile of relative PATE mRNA abundance in developing embryos was demonstrated by RT-PCR (Figure 8B), suggesting that the developmental change in PATE activity was due to a change in PATE gene expression. The 539-bp actin RT-PCR product corresponds to a fragment amplified from constitutive cotton actin mRNA to help evaluate the amounts of total RNA template used in RT-PCR reactions. Similar results were obtained with slot blots of total RNA probed with random-primed DNA fragments derived from cotton PATE cDNA and the actin RT-PCR fragment (Figure 8C), with the relative PATE/actin ^{32}P dpm ratio being slightly higher for 28 dpa embryos (PATE/actin ratio of 0.56 relative to an average ratio of 0.42 for the others).
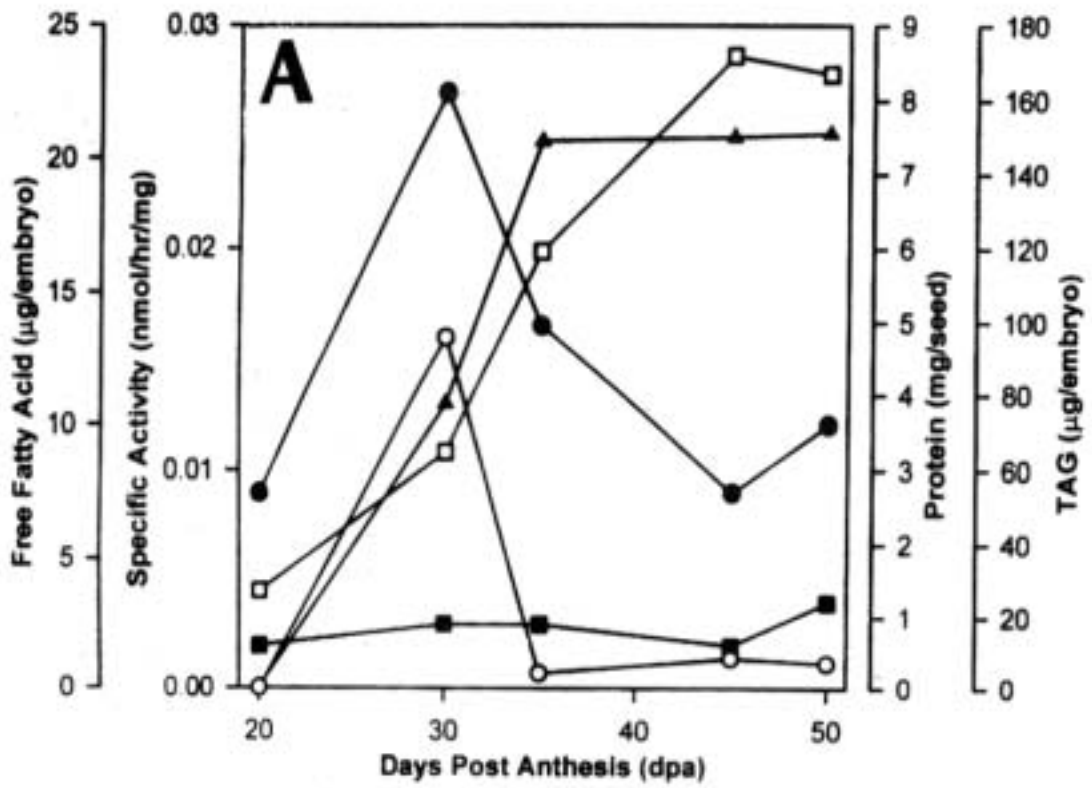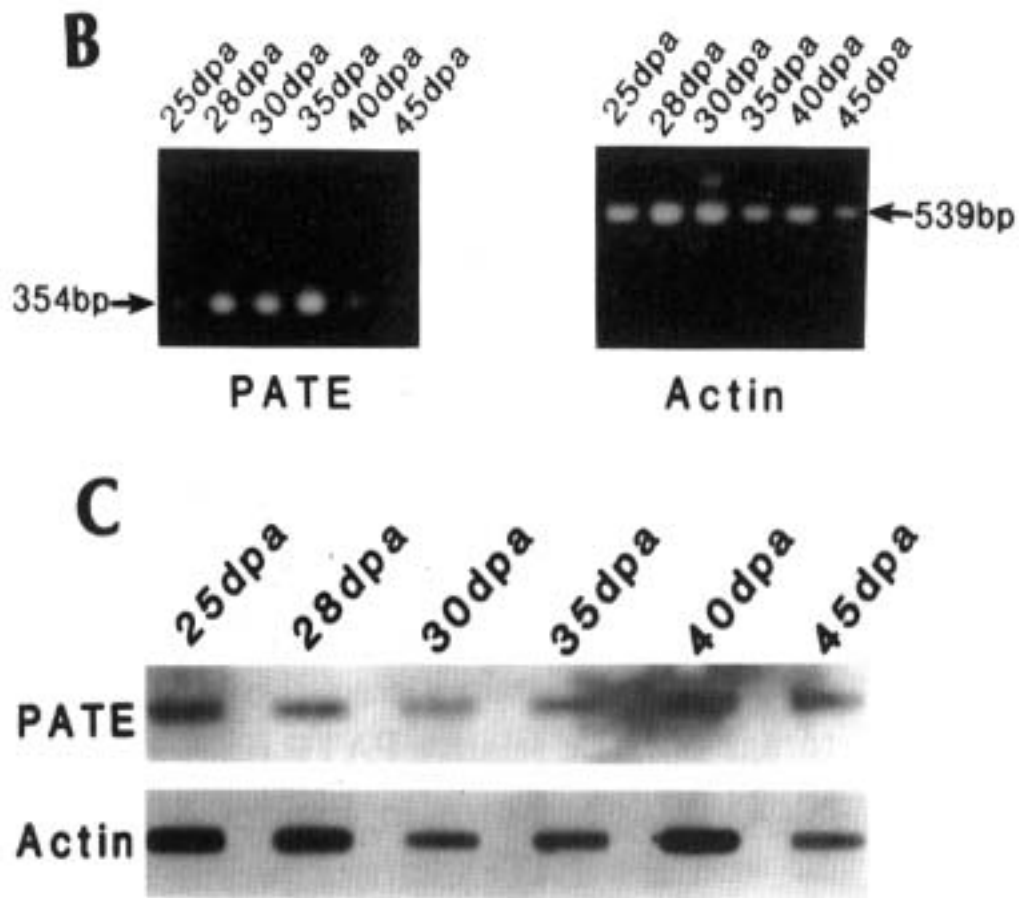
Figure 8. Developmental expression of thioesterase activity in cotton embryos. (A)
Developmental profile of thioesterase specific activity, free fatty acid content,
triacylglycerol content and protein content in cell-free extracts from embryos excised
from bolls at various stages post anthesis. Thioesterase activity was measured toward
[^{14}C]-palmitoyl-ACP (solid circles) or [^{14}C]-oleoyl-ACP (solid squares) synthesized and
purified as described in Methods. Values are representative of a single experiment with

similar trends evident in three independent experiments. The free fatty acid content is

shown by open circles, the triacylglycerol (TAG) levels by solid triangles, and the protein

concentrations by open squares. (B)   The developmental expression of cotton PATE and

actin transcript abundance was evaluated by relative RT-PCR with 0.2 µg total cotton

RNA in each reaction for each developmental stage with PATE forward and reverse PCR

primers (Fig. 1) and actin forward and reverse PCR primers. The expected PATE and

actin RT-PCR products of 354 bp and 539 bp, respectively, were visualized with

ethidium bromide staining following electrophoresis in 3% agarose gels. (C) Slot-blot

hybridization of 10 µg total cotton RNA isolated from embryos at different stages

postanthesis with homologous ^{32}P-labeled cotton PATE and actin probes generated by

random priming (slot blots were conducted in Dr. Pirtle's Laboratory with the assistance

of Irma Pirtle).

Discussion

Functional and structural differences occur between the plant FatA and FatB acyl-ACP thioesterase enzymes (Jones et al., 1995; Voelker, 1996). On a functional basis, the FatA thioesterases have a substrate specificity especially for oleoyl-ACPs, whereas the FatB thioesterases exhibit a wider latitude of substrate specificities, albeit toward saturated acyl-ACPs, of different chain lengths. The FatB enzymes can be further subdivided into two groups. One group has a greater specificity for hydrolyzing short/medium-chain C8-C12 acyl-ACPs, similar to the acyl groups in endogenous seed oil TAGs in species like *Cuphea* and *Lauraceae* (California bay). The other group has a greater specificity for hydrolyzing long-chain C14-C18 acyl-ACPs, especially palmitoyl-ACPs, with their gene expression probably occurring in all major plant tissues. On a structural basis, the predicted amino acid sequences of FatA and FatB thioesterase preproteins have three major sequence-length variations, as observed by Jones et al. (1995) and reviewed by Voelker (1996), shown by the dashed-line gaps around residues 90, 290, and 390 in Figure 3. Relative to the predicted amino acid sequences of the FatB preproteins, the FatA cDNAs encode preproteins lacking a hallmark Fat-class indicator domain of about 25 hydrophobic amino acids occurring in the FatB preproteins. From a comparison of the predicted mature polypeptide sequences of 34 thioesterases (devoid of the highly variable N-terminal stroma-targeting domains), Voelker (1996) generated a phylogenetic tree with two major branches separating the FatA and FatB sequences, most likely meaning that the FatA and FatB genes are paralogous from an ancient gene duplication, signifying that there are two conserved and distinct thioesterase classes in all

38

plant species with different functional activities. Jones et al. (1995) isolated a FatB palmitoyl-ACP thioesterase cDNA from *Cuphea hookerenia* seeds that have TAGs with predominantly C8 and C10 acyl chains. This observation strongly supports the hypothesis that higher plant tissues contain both an oleoyl-ACP thioesterase activity and a less specific palmitoyl-ACP thioesterase activity. This is similar to an earlier suggestion by Browse and Somerville (1991) for the occurrence of a second plant acyl-ACP thioesterase other than the oleoyl-ACP thioesterase.

Dörmann et al. (1995) analyzed an *Arabidopsis* cDNA clone designated TE 3-2 encoding the first FatB long-chain acyl-ACP thioesterase, with highest activity towards palmitoyl-ACP when expressed in *E. coli*, but still with significant activities for other long-chain acyl-ACPs. They also analyzed another *Arabidopsis* cDNA clone designated TE 1-7 that appeared to encode the *Arabidopsis* FatA oleoyl-ACP thioesterase, since it was 60% identical to other FatA oleoyl-ACP thioesterases.   The clone TE 3-2 seems to encode an enzyme with a broader specificity for C14-C18-ACPs than the oleoyl-ACP thioesterase, and must encode the *Arabidopsis* FatB thioesterase, due to its functional activity profile, its constitutive expression in all plant tissues, and its maximal expression around 20-30 days after flowering, correlating well with TAG synthesis in seeds.

The putative 413-residue cotton PATE preprotein would indeed be a member of the FatB class, since it has greater identities with the 412-amino acid *Arabidopsis* and the 416-residue *Cuphea hookeriana* preproteins than with polypeptides of the FatA class, such as the predicted 362-amino acid *Brassica rapa* preprotein (Jones et al., 1995; GenBank accession U17098). The presumptive cotton PATE polypeptide has the three

length variations seen in FatB thioesterases, including the hallmark hydrophobic stretch of N-terminal amino acids (residues 84-110 in Figures 2 and 3), leading to the conclusion that this cotton cDNA clone does indeed encode a FatB1 thioesterase preprotein.   The likely transit peptide cleavage site for production of the mature cotton PATE protein would be L84 (shown in Figure 2), when compared with other thioesterase preprotein sequences (Gavel and von Heijne, 1990; Dörmann et al., 1995; Cline and Henry, 1996). The conserved active-site catalytic cysteine required for formation of the covalent thioester enzyme intermediate and the histidine essential for general base catalysis in the plant acyl-ACP thioesterases (Yuan et al., 1996) likely correspond to the homologous residues C312 and H347 in the cotton PATE amino acid sequence.   Two other conserved histidine residues also occur in the plant acyl-ACP thioesterases (Yuan et al., 1996), and would correspond to the homologous H167 and H377 residues shown in Figure 2.

There is only one EcoRI cleavage site (at residue 816) and no HindIII cleavage sites within the 1.7-kb cDNA sequence in Figure 2. At least two EcoRI fragments and one HindIII fragment from the cotton genomic DNA around the PATE gene would be detected by hybridization with fragments derived from the 1.4-kb Arabidopsis probe. As can be seen from the genomic blot in Figure 4, there are four hybridizing EcoRI fragments, two of which must be derived from the PATE gene due to the position of the EcoRI cleavage and the extent of the region covered by the probe. Thus, there must be at least two PATE genes in the cotton genome, and this conclusion is supported by the presence of four hybridizing HindIII fragments.

Further evidence for the existence of a FatB1 thioesterase in cotton with a substrate preference for palmitoyl-ACP is provided by the tissue and developmental profiles of endogenous thioesterase enzyme activity and the RT-PCR and slot-blot analyses of relative PATE transcript (Figures 7 and 8). As anticipated, the highest specific activity toward palmitoyl-ACP is in embryos, in which TAG synthesis is the greatest, while activity toward oleoyl-ACP is surprisingly eight-fold less in embryos (Figure 7A). In contrast, the activities towards both acyl-ACPs is least in mature leaves. The relative PATE transcript abundance, as detected by RT-PCR (Figure 7B) and slot-blot hybridization (Figure 7C) appears to be more abundant in embryos than in hypocotyls, roots, cotyledons, or mature leaves. As shown in Figure 8, the specific activity toward palmitoyl-ACP is maximal about 30 dpa while that toward oleoyl-ACP is much less, but nonetheless fairly constant during development. The TAG levels are highest just after the PATE activity, most likely from the incorporation of palmitic acid (and other fatty acids) into storage oil. The relative PATE transcript abundance around 28-30 dpa shown in Figures 8B and 8C coincides well with the PATE activity profile. In other work, endoplasmic reticulum lysophosphatidic acid and diacylglycerol acyltransferase activities exhibited a developmental profile similar to PATE, peaking at the maximum stage of cottonseed oil biosynthesis (Turley and Chapman, 1998). It would seem that mRNA levels and enzyme activity levels are well coordinated for maximal expression during seed development in cotton plants. Consequently, PATE expression represents one potential target for the metabolic engineering of palmitic acid levels in cotton seed.

Acknowledgements

References

Anderson MLM, Young BD (1985) Quantitative Filter Hybridization. *In* Nucleic Acid Hybridization: A Practical Approach. Hames BD, Higgins SJ (eds.) IRL Press, Washington, D.C., pp. 73-111.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds. (1987) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York. pp. 2.5.5, 6.0.1-6.3.6.

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

Chapman KD, Sprinkle WB (1996) Developmental, tissue-specific, and environmental factors regulate the biosynthesis of N-acylphosphatidylethanolamine in cotton (*Gossypium hirsutum* L.). *J. Plant Physiol.* 149: 277-284.

Chapman KD, Trelease RN (1991) Acquisition of membrane lipids by differentiating glyoxysomes: role of lipid bodies. *J. Cell Biol.* 115: 995-1007.

Christie WW (1982) *Lipid Analysis, 2nd ed.* Pergamon Press, New York. pp. 52-54.

Cline K, Henry R (1996) Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell Dev. Biol.* 12: 1-26.

Dehesh K, Edwards P, Hayes T, Cranmer AM, Fillatti J (1996) Two novel thioesterases are key determinants of the bimodal distribution of acyl chain length of *Cuphea palustris* seed oil. *Plant Physiol.* 110: 203-210.

Del Vecchio AJ (1996) High-laurate canola. *Inform* 7: 230-243.

Dörmann P, Voelker TA, Ohlrogge JB (1995) Cloning and expression in *Escherichia coli* of a novel thioesterase from *Arabidopsis thaliana* specific for long-chain acyl-acyl carrier proteins. *Arch. Biochem. Biophys*. 36: 612-618.

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem*. 132: 6-13.

Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett.* 261: 455-458.

Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochim. Biophys. Acta* 1301: 7-56.

Hunt AG (1994) Messenger RNA 3' end formation in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45: 47-60.

Jones A, Davies HM, Voelker TA (1995) Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. *Plant Cell* 7: 359-371.

Kinney AJ (1997) Genetic engineering of oilseeds for desired traits. *In* Genetic Engineering, vol. 19. Setlow JK (ed.) Plenum Press, New York. pp. 149-166.

Ni W, Trelease RN (1991) Two genes encode the two subunits of cottonseed catalase. *Arch. Biochem. Biophys.* 289: 237-243.

Ohlrogge J, Browse J (1995) Lipid biosynthesis. *Plant Cell* 7: 957-970.

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 109-136.

Paterson AH, Brubaker CL, Wendel JF (1993) A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. *Plant Mol. Biol. Reporter* 11: 122-127.

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

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucl. Acids Res*. 13: 7207-7221.

Sagerstrⴑm CG, Sive HL (1996) RNA Blot Analysis. *In* A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis. Krieg PA (ed.) Wiley-Liss, New York. pp. 83-103.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seidman CE, Struhl K, Sheen J, Jessen T (1997) Introduction of Plasmid DNA into Cells. *In* Current Protocols in Molecular Biology. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, Albright LM, Coen DM, Varki A, Janssen K (eds.) John Wiley and Sons, Inc., USA. pp.1.8.1-1.8.10.

Turley RB, Chapman KD (1998) Ontogeny of cottonseeds: gametogenesis, embryogenesis, germination, and seedling growth. *In* Cotton Physiology - Book II, Chapter 38. Steward M, Oosterhuis D, Heitholt J (eds.) National Cotton Council, Memphis, TN.

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

Voelker T (1996) Plant acyl-ACP thioesterases: Chain-length determining enzymes in plant fatty acid biosynthesis. *In* Genetic Engineering, Vol. 18. Setlow JK (ed.) Plenum Press, New York. pp. 111-133.

Voelker T A, Jones A, Cranmer AM, Davies HM, Knutzon DS (1997) Broad-range and binary-range acyl-acyl-carrier-protein thioesterases suggest an alternative mechanism for medium-chain production in seeds. *Plant Physiol*. 114: 669-677.

Wan CY, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem*. 223: 7-12.

Wilkins TA, Smart LB (1996) Isolation of RNA from plant tissue. *In* A Laboratory Guide to RNA Isolation, Analysis and Synthesis. Kreig PA (ed.) Wiley-Liss, New York. pp. 21-41.

Yuan L, Nelson BA, Caryl G (1996) The catalytic cysteine and histidine in the plant acyl-acyl carrier protein thioesterases. *J. Biol. Chem*. 271: 3417-3419.

CHAPTER 2

EXPRESSION OF THE COTTON *FATB* cDNA IN *ESCHERICHIA COLI*

Portions of this chapter were accepted for publication in Plant Physiology and

Biochemistry (2001); Expression of a Cotton (*Gossypium hirsutum* L.) cDNA Encoding

a FatB Palmitoyl-Acyl Carrier Protein Thioesterase in *Escherichia coli*; Tu T. Huynh,

Robert M. Pirtle, and Kent D. Chapman

Abstract

A cotton *FatB* cDNA (isolated and sequenced in Dr. Pirtle's Laboratory)

encoding a palmitoyl-acyl carrier protein (ACP) thioesterase (Genbank Accession

#AF034266) was expressed in various *E. coli* strains. Transcription and translation in a

coupled *in vitro* system revealed the presence of two [^{35}S-Met]-labeled protein products,

one of about 35 kDa and one of about 46 kDa. The 46 kDa polypeptide likely

represented the translation of the preprotein while the 35 kDa polypeptide likely

represented a translation product initiated at an alternative, internal in-frame initiation

codon. Polyclonal anti-peptide antibodies were used to confirm the accumulation of this

truncated protein. An immunoreactive 35 kDa protein was recognized in transformed *E.

coli* cell lysates supporting the notion that indeed there was an internal start site, which

seemed to be preferred when the cotton cDNA was expressed in *E. coli*. In crude

homogenates of cotton embryos (30 dpa, days post anthesis) and cotyledons of 48 h dark-grown seedlings, a 37 kDa protein, which likely represents the mature processed FatB protein, was recognized. When acyl-CoA synthetase - minus *E. coli* mutants (K27 *fad*D88 mutant, CGSC #5478) were transformed with the cotton *FatB* cDNA, a four- to five-fold increase in 16:0 free fatty acid content was measured in the culture medium. Acyl-ACP thioesterase activity assays in *E. coli* lysates revealed that there was a clear preference for palmitoyl-ACP over oleoyl-ACP *in vitro*. Collectively, our results indicate that indeed the cotton *FatB* cDNA encodes a functional thioesterase with a preference for saturated acyl-ACPs (FatB) over unsaturated acyl-ACPs (FatA).

Abbreviations

ACP, acyl carrier protein; bp, base pair(s); dpa, days post anthesis; FAS, fatty acid synthase; GC-FID, gas chromatography - flame ionization detection; kDa, kilodaltons; nt, nucleotide(s); ORF, open reading frame; PATE, palmitoyl-acyl carrier protein (ACP) thioesterase; RT-PCR, reverse-transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; TLC, thin layer chromatography; UTR, untranslated region.

Introduction

The fatty acid biosynthetic pathway is a primary metabolic pathway found in every cell and is essential for growth (Ohlrogge and Jaworski, 1997). This pathway consists of the sequential condensation of two carbon units onto a growing fatty acyl chain, which is attached to an acyl- carrier protein (ACP). Four specific reactions are required for the extension of the acyl chains; enzymes involved in the condensation-reduction-dehydration-reduction cycle can reside on multifunctional polypeptides or as individual entities of the fatty acid synthase (FAS) (McCarthy and Hardie, 1984). Plants synthesize fatty acids through a series of discrete enzymes localized to plastids (Browse and Somerville, 1991). In *Escherichia coli* and other prokaryotes, each FAS reaction is catalyzed by a discrete, monofunctional enzyme as well. This type of FAS complex has been termed the prokaryotic, or type II dissociable enzyme complex (Ohlrogge and Browse, 1995; Harwood, 1996; Kinney, 1997; Ohlrogge and Jaworski, 1997). It has been reported that the plant and prokaryotic FASs resemble each other in structure (Ohlrogge et al., 1979; Harwood, 1996). The structural similarities likely are due to a common ancestry of plant and prokaryotic FASs (Ohlrogge, 1982), consistent with the plastidic location of *de novo* fatty acid synthesis in plants. By contrast, in mammals and yeast the non-dissociable enzymatic activities reside on one or two multifunctional polypeptides (McCarthy and Hardie, 1984), designated the eukaryotic or type I fatty acid synthase.

Acyl-ACP thioesterases terminate acyl-chain elongation during fatty acid biosynthesis (Harwood, 1988). A number of studies have focused on investigating how chain length is determined (Browse and Somerville, 1991; Ohlrogge et al. 1993; Voelker

49

1996). Currently, it is believed that the substrate specificities of the acyl-ACP

thioesterases play a major role in determining when the acyl chains are cleaved and

released from the ACPs, and thus directly influence the pools of free fatty acids available

for export to extraplastidic compartments. Acyl-ACP thioesterase cDNAs from a number

of plant species have been isolated (Voelker, 1996; Yoder et al., 1999). The plant acyl-

ACP thioesterases are usually categorized into two groups depending on sequence

identity and, in some cases, acyl-ACP preference. Jones et al. (1995) originally classified

the two different types of thioesterases as FatA or FatB, with substrate preferences for

unsatuarated acyl-ACPs or saturated acyl-ACPs, respectively. Unlike the FatA

thioesterases which prefer specifically oleoyl-ACPs, the FatB thioesterases show a vast

array of specificities and are further divided into subgroups depending on their preference

for saturated substrates of various chain lengths (Voelker, 1996). For example, Dehesh et

al. (1996) reported the isolation of two thioesterase cDNAs from *Cuphea palustris* with

different substrate specificities. When expressed in *E. coli*, the *Cuphea palustris* FatB1

thioesterase preferred the short chain acyl-ACPs (C8-C10 acyl-ACPs) while the *Cuphea*

palustris FatB2 thioesterase preferred medium chain acyl-ACPs (C14-C16 acyl-ACPs).

Expression of other thioesterases in *E. coli* also revealed substrate preferences for longer

chain acyl-ACPs (C16-C18 acyl-ACPs), such as the *Arabidopsis* FatB1 thioesterase

(Dörmann et al., 1995) and the *Cuphea hookeriana* FatB1 thioesterase (Jones et al., 1995;

Voelker, 1996).

Expression of acyl-ACP thioesterases in *E. coli* has posed problems in some

strains, perhaps by interfering with endogenous *E. coli* FAS. Dörmann et al. (1995)

reported some difficulties with the growth of the temperature-sensitive transformed cells

and the toxicity of the accumulated, enzymatically active recombinant protein.

Expression of the mature polypeptide in *E. coli* was apparently toxic to the bacterial cell,

but it is unclear whether this was due to the accumulation of the recombinant protein or

release of excess free fatty acids. Because free fatty acids are not normally found in *E.*

coli as intermediates of lipid biosynthesis (Voelker and Davies, 1994), it is possible that

the expression of acyl-ACP thioesterases in *E. coli* increased the amounts of free fatty

acids to toxic levels. Furthermore, expression of a full-length thioesterase cDNA will

result in the translation of a protein with a stromal targeting domain. The insertion of this

domain into *E. coli* membranes may cause the *E. coli* cells to overaccumulate inclusion

bodies and become unstable.

In attempts to characterize functionally a cotton *FatB* cDNA (isolation described

in Pirtle et al., 1999), we have expressed this cDNA in various *E. coli* strains and

examined its thioesterase activity *in vivo* and *in vitro*. The predicted amino acid sequence

of the cotton palmitoyl-ACP thioesterase (PATE) preprotein has a 63% identity to the

Arabidopsis long-chain acyl-ACP thioesterase FatB, a 54% identity to *Cuphea*

hookeriana FatB, and only a 22% identity to the *Brassica* FatA preprotein. Thioesterase

activity assays in crude cotton homogenates suggested that in some organs (e.g.

cotyledons of cotton seedlings and 30 dpa developing embryos), a FatB1-like enzyme

predominated (Pirtle et al., 1999). In this chapter, I present results which suggest that

indeed the cotton *FatB* cDNA encodes a functional thioesterase with a preference in *E. coli* cells and in cell-free lysates for saturated acyl-ACPs, and may indicate a primary role for this enzyme in production of extraplastidial, saturated fatty acids in some cotton tissues.

Materials and Methods

Plant Material and *E. coli* Strains

Cotton (*Gossypium hirsutum* L., cv. Paymaster HS26) plants were grown in the greenhouse under conditions described previously (Chapman and Sprinkle, 1996). Developing bolls were collected at 30 days post anthesis (dpa) from mature plants (flowers tagged at anthesis). The embryos were excised from ovules and frozen in liquid nitrogen and stored at -80°C for protein extractions and Western blot analyses. Cotyledons were collected from seedlings germinated at 30°C and grown in the dark for 48h. Seeds were a gift from Dr. John J. Burke (USDA-ARS, Lubbock).

Various *E. coli* strains were used in subsequent experiments. Top10™ (Invitrogen) and JM109 (Promega) *E. coli* cells were the hosts for plasmid production: Plasmid DNA from these strains were templates for coupled *in vitro* transcription and translation experiments. SolR (Stratagene) and K27 *fad*D88 (acyl-CoA synthetase minus mutants; *E. coli* Genetic Stock Center, Yale University, CGSC #5478) *E. coli* cell lysates were used in thioesterase assays. Cell lysates from the *fad*D88 mutant also were used for immunoblotting. All *E. coli* strains were grown in LB media (1% Bacto Tryptone, 0.5%

Bacto Yeast Extract, 1% NaCl , pH 7.0). For plasmid selection, 500 µg/mL of ampicillin was added. Liquid cultures were shaken at 200 rpm overnight (37°C), except the *fad*D88 mutant which was grown at 30°C.

Previous studies with plant thioesterases report that the expression of acyl-ACPs thioesterases in *E. coli* are toxic to the bacterial cells (Dörmann et al., 1995). The toxicity of the expressed thioesterases may be due to the excess amounts of free fatty acids in the *E. coli* cells. Free fatty acids do not exist as lipid intermediate in *E. coli*. Thus the excess amounts of free fatty acids can act as detergents that solubilize the bacterial membranes. Another possibility for the toxicity may be due to the translation of the full-length thioesterase proteins, which have 5'-terminal stromal targeting domains (STD). These STDs may insert into the bacterial membranes resulting in the over-accumulation of inclusion bodies, which cause the bacterial cells to become unstable. To avoid problems with the toxicity of expressing the cotton *FatB* cDNA in *E. coli*, I used an acyl-CoA synthetase minus *E. coli* mutant (*fadD*88). These mutants lack the acyl-CoA synthetase enzyme and thus cannot utilize the free fatty acids produced by the recombinant FatB thioesterase. Instead these mutants secrete the free fatty acids into the culture medium, allowing the possibility of measuring the *in vivo* thioesterase activity.

E. coli Transformation and Cloning

Primers were designed to obtain full-length *PATE* RT-PCR products from total RNA of 30 dpa embryos and cotyledons of 2 d-old cotton seedlings (Pirtle et al, 1999). The *PATE* amplimers (Biosynthesis, Inc.; Lewisville,TX) for PCR amplification were 5'-

CCATGGTTGCTACTGCT-3' (forward) and 5'-AAACAAGCCTAATTACAACC-3'
(reverse). The forward amplimer was designed to include 2 nucleotides from the 5'
flanking non-translated region while the reverse amplimer was designed within the
coding sequence of the cDNA sequence (Genbank accession number AF034266). The
1370-bp RT-PCR product was blunt end-ligated into the *Eco*RV site of pZerO-2.1
(Invitrogen) and transformed into Top10 *E. coli* cells with the Fast-link DNA Ligation
and Screening Kit from Epicentre. Plasmid DNA was isolated with the Promega Wizard
Pkus SV DNA Purification System and the *PATE* cDNA insert sequenced was verified
completely (Epicentre SequiTherm EXCEL II Long-Read DNA Sequencing Kit-LC) on a
LiCOR 4000L semi-automated sequencer (Lincoln, NE). *PATE* primers also were
designed for incorporation of flanking restriction sites for directional cloning into *E. coli*
expression vectors. The sequences of the primers (Biosynthesis, Inc.) with added *Xba*I
and *Sac*I restriction sites were 5'-CGTCTAGAATGGTTGCTACTGCTGTGACATCG-
3' (forward) and 5'-CCGAGCTCCTTGGATTTCTATGCCCTTTTTGCG-3' (reverse),
respectively.

The amplified fragment was ligated into the *Xba*I and *Sac*I sites of pGEM-7Zf(+)
(Promega) and the resulting construct designated pGEMPATE6 was transformed into
Top10 *E. coli* cells. pGEMPATE6 Plasmid DNA was isolated as above and used as
template for *in vitro* transcription/translation experiments. The *PATE* cDNA insert in
pGEMPATE6 was excised from pGEM-7Zf(+) with *Xba*I and *Sac*I restriction enzymes
and directionally subcloned into the *Xba*I and *Sac*I restriction sites in the multiple cloning
site of pUC19 (Gibco BRL). The subclone was designated pCPATE6. A 13-mer linker,

5'-AGCTCGCCAGGCG-3' (Biosynthesis, Inc.), was ligated into the *Hind*III site prior to transformation into Top10, *fad*D88 mutant, and JM109 *E. coli* cells. The linker placed the ATG initiation codon of the *PATE* coding region in-frame relative to the ATG initiation codon of the *lacZ* α-peptide (truncated at amino acid 6) in the pCPATE6 vector.

Transcription and Translation *In vitro*

The pCPATE6 and pGEMPATE6 clones were used as the DNA template (2 μg) for transcription and translation *in vitro* using the Promega *E. coli* S30 extract. The reaction mixture contained the S30 extract with 10 μCi of ^{35}S-Met (ICN Biomedicals, Inc.; 1175 Ci/mmol) and was done according to the manufacturer's protocol. The radio-labeled translation products were acetone precipitated and separated by SDS-PAGE. After electrophoresis, the gel was stained in 0.25% (w/v) Coomassie Brilliant Blue R250 (Sigma), 45% (v/v) methanol, and 10% (v/v) glacial acetic acid at room temperature overnight and destained in 45% (v/v) methanol and 10% (v/v) acetic acid for 1 h. The gel was washed in 3% (v/v) glycerol, 40% (v/v) methanol, and 10% (v/v) acetic acid for 1 h and Entensify Universal Autoradiography Enhancer (Dupont) for 2 h prior to drying on a Biorad Model 583 Gel Dryer for 60 min at 60°C. The dried gel was exposed to X-OMAT-AR film (Kodak) overnight at –80°C with intensifying screens (Fisher FBIS 810).

Preparation of cotton cell-free extracts and *E. coli* lysates

Cell free homogenates of 30 dpa embryos and cotyledons of germinated cotton seedlings were prepared as described previously (Pirtle et al., 1999) and boiled in 1:1 (v/v) 2X sample buffer (Speicher, 1999) for SDS-PAGE.

Lysates of transformed *E. coli* SolR cells expressing the cotton *PATE* cDNA designated SKCPc115b (Yoder et al., 1999) as a *lac*Z-PATE fusion polypeptide were prepared according to the methodology of Pappan et al. (1997) with modifications to assess the thioesterase activity of the *lac*Z-PATE fusion polypeptide. The bacterial cells were incubated in LB medium containing 50 μg/mL ampicillin at 37°C with shaking. Isopropyl 8-D-thiogalactopyranoside (IPTG, 2 mM) was added to the medium after 3 h and the cells were grown overnight at 30°C without shaking. The cells were pelleted at 6500 x*g* for 10 min and resuspended in 3 mL of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed with sonication (Microson ultrasonic cell disruptor with CM-2 converter probe, setting at 1). Cell debris was removed by centrifugation and the supernatants were analyzed for protein content and thioesterase activity.

Lysates of transformed *E. coli* K27 *fadD*88 cells carrying the *PATE* cDNA in pCPATE6 were prepared with B-Clear II bacterial cell lysis extraction reagent (Sigma). The soluble and wash fractions were collected as described in the manufacturer's protocol. The fractions were immediately used in thioesterase assays or boiled in 2X SDS-sample buffer (Speicher, 1999) for immunoblotting.

Antibody production and immunoblots

56

Polyclonal anti-peptide antibodies commercially generated (Alpha Diagnostics) were directed against amino acid residues 395-412 (RPKHAKSSANMDQITAKR) near the C-terminus of the deduced amino acid sequence for the cotton *PATE* cDNA clone SKCPc115b (Yoder et al., 1999; Genbank Accession number AF034266).

To confirm that the antibodies specifically recognize the peptide fragment, 5 μg of the peptide used to generate anti-FatB antibodies (Alpha Diagnostic) was hydrated in 10% acetic acid and spotted onto PVDF (polyvinylidene difluoride) membrane for subsequent incubation with the FatB antibodies. The fourth bleed serum was diluted 1:1,000, 1:5,000, and 1:10,000. Membranes incubated with the 1:5,000 diluted anti-FatB serum gave the strongest immunoreaction (with low background, non-specific interactions) and used in all subsequent immunoblots. In addition, the secondary antibody (goat anti-rabbit IgG/alkaline phophatase; New England Biolabs) did not non-specifically bind to the peptide fragment. Membranes incubated with the secondary antibodies alone (without anti-FatB antibodies) resulted in no immunoreaction with the peptides.

Protein from crude homogenates and *E. coli* lysates were separated by Tris-SDS-PAGE (12%T/0.3%C resolving gel and 3.9%T/0.3%C stacking gel) in a Mini-Protean II Cell (Bio-Rad) according to the method of Laemmli (1970). Briefly, cast gels were pre-electrophoresed in 25 mM Tris (pH 8.3), 200 mM glycine, 1% (w/v) SDS, and 1mM thioglycolate for 45-60 min. at 50 volts (Microprocessor Controlled Electrophoresis FB703, FisherBiotech). The same buffer without thioglycolate was replaced prior to

loading of protein samples. Protein samples dissolved in SDS-treatment buffer (above) were electrophoresed at 30 volts until the samples were fully within the stacking gel (30-45 min.). The voltage was then gradually increased by maintaining constant current (75mA) until the dye front reached the bottom of the gel (~2 h). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Biorad) via semi-dry electroblotting (Schãgger, 1994) in a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell with the current limited to 100 mA/gel ($5.5mA/cm^{-2}$). Transfer time was typically 1 h and proteins immobilized on the PVDF membrane were incubated in 20 mM Tris, pH 7.5, 500mM NaCl, 0.1% Tween-20 and 3% (w/v) defatted BSA prior to antibody incubations. Monoclonal antisera directed toward a 18-mer peptide of the cotton preprotein (above) was diluted 5,000 fold and antigen recognition was visualized by Alkaline Phosphatase Color Development Reagents (Bio-Rad), BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt) and NBT (*p*-nitro blue tetrazolium chloride), with goat anti-rabbit IgG/alkaline phosphatase conjugate diluted 1:5000 (New England Biolabs).

Release of fatty acid by *E. coli* mutants

Cultures (9 mL) of *E. coli* K27 *fadD*88 cells without or with pCPATE6 DNA were grown overnight at 30°C to an absorbance of 0.9 at 600nm and total lipids were extracted from the media as described by Voelker and Davies (1994). As an internal standard, 0.2 mg of heptadecanoic acid was added to the cultures prior to total lipid extractions. Free fatty acids were separated from polar lipids by thin-layer

chromatography with hexane/diethylether/acetic acid (60/40/1) (Dörmann et al, 1995).

Free fatty acids were extracted from the appropriate regions of separate TLC plates and

transesterified (Christie, 1982). The fatty acid methyl esters were quantified by gas

chromatography-flame ionization detection (GC-FID) on a Hewlett-Packard 5890 Series

II Gas Chromatograph using a 30 M DB-23 capillary column (0.25 mm i.d., 0.25 μm film

thickness) and isothermal elution at $200^{o}C$ (both injector and detector set at $250^{o}C$) and

15 psi with N_2 as the carrier gas.

Thioesterase activity assays

Acyl-ACPs (*E. coli* ACP, Sigma) were synthesized as described previously

(Chapter 1; Pirtle et al., 1999), except purified *E. coli* acyl-acyl carrier protein synthetase

(Shanklin, 2000) was used (Gift from Dr. John Shanklin, Brookhaven National

Laboratory). The thioesterase enzyme reactions were initiated with the addition of the

$[^{14}C]$-acyl-ACP (60 μM, about 2200 dpms) to the *E. coli* lysates (500 μg) of

nontransformed or transformed lines. Assays were carried out as previously described

(Pirtle et al., 1999) except the reactions were terminated after 1 h.

To confirm that the activities in the *E. coli fad*D88 mutant cell lysates were not

contaminated with acyltransferase activity, two fractions were collected when the lysates

were prepared. Using Sigma B-Clear II bacterial cell lysis extraction reagent, the

supernatant collected after the lysis of the *E. coli* cells were referred to as the soluble

protein fraction. The remaining pellet was incubated with lysozyme according to the

manufacturer's protocol. Released membrane-bound proteins were then collected in the

second supernatant. Thioesterase assays were conducted as described and total lipids were extracted from the reaction mixture. The free fatty acids were separated from the polar lipids on TLC as described above. Radioactivity in the polar lipid and free fatty acid regions on the TLC plate were analyzed by radiometric scanning (Bioscan System 200) and converted to acyltransferase or thioesterase activities, respectively.

Results

Expression of the Full-length cotton FatB cDNA clone

To generate a full-length cotton FatB cDNA clone, unique oligonucleotide primers (see Materials and Methods) were designed for the 5' and 3'- untranslated regions (UTRs) from the FatB gene sequence (Yoder et al., 1999). A 1370-bp RT-PCR product was amplified from total RNA of embryos (30 dpa) and confirmed by DNA sequence analysis to encompass an ORF of 1242-bp. Expression of the full-length cDNA would generate a preprotein of 413 amino acids (Figure 1), with a predicted molecular weight of 46 kDa. Proteolytic processing of the preprotein at L84, the putative transit peptide cleavage site (Figure 1), would generate the mature form of the protein of 329 amino acids with a predicted molecular weight of 37 kDa. The RT-PCR product was directionally subcloned into pUC19 and transformed into several *E. coli* strains for expression of the full-length *FatB* cDNA clone. A linker-adaptor was inserted into the *Hind*III site of the vector such that the translational start of the FatB sequence was in-frame with the translational start of the *lac* Z-α polypeptide. The resulting plasmid construct was designated pCPATE6.
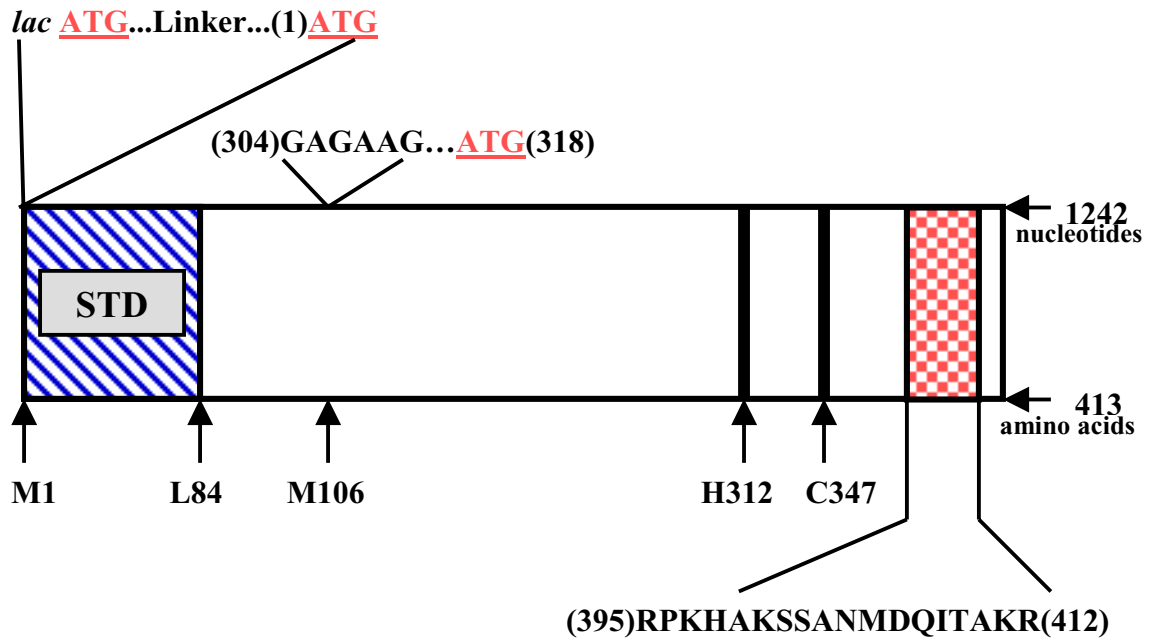
Figure 1: Simplified diagram highlighting some features of the cotton *PATE* cDNA

clone SKCPc115b (Yoder et al., 1999; Genbank Accession #AF034266), which has a

coding region of 1242 nucleotides (numbers above the bar) for 413 amino acids (numbers

below the bar). The hatched region labeled STD represents the stromal targeting domain.

The putative transient peptide cleavage site is probably L84 (Gavel and von Heijne, 1990;

Dörmann et al., 1995; Cline and Henry, 1996). The first methionine (M) of the

preprotein is designated as M1 and an in-frame potential internal initiation codon is

designated as M106. There is a Shine-Dalgarno consensus ribosome binding sequence

GAGAAG upstream from this potential internal start site (at 304 bp). A linker was

inserted upstream of M1 such that the start site of the preprotein is in-frame with the start site of the pUC19 vector *lacZ* α-polypeptide. The amino acid residues reported to be involved in catalysis are H312 and C347, which are designated with solid black bars and are conserved (Yuan et al., 1996; Pirtle et al., 1999). The region chosen for anti-peptide antibody production (amino acid residues 395-412) is located within the checkered region.
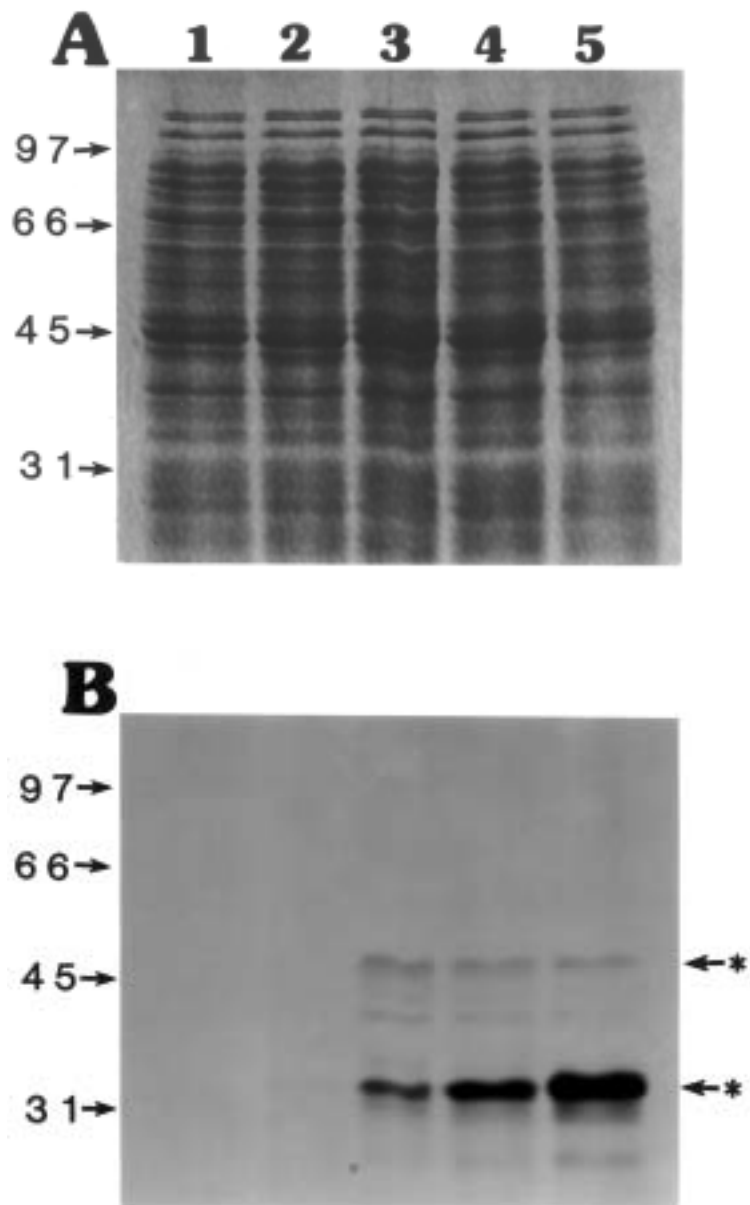
Figure 2: Protein profiles (including [^{35}S-Met]-labeled translation products) of *in vitro* transcription/translation reactions with no DNA template (lane 1), with pGEMPATE6

plasmid DNA (insert in reverse orientation relative to the *lac*Z promoter, lane 2), with

pCPATE6 plasmid DNA propagated in JM109 (lane3), with pCPATE6 plasmid DNA

propagated in Top10^{TM} (lane 4), with pCPATE6 plasmid DNA propagated in Top10^{TM} +

4:M PMSF (lane 5). **A.** Coomassie-stained SDS-PAGE gel of acetone-precipitated

proteins from the reactions above. **B.** Autoradiogram of the dried gel. Asterisks

represent a 46 kDa [^{35}S-Met]-labeled translation product and a 35 kDa [^{35}S-Met]-labeled

translation product. Molecular weights of standard proteins are indicated on the left in

kDa.

pCPATE6 plasmid DNA amplified in various *E. coli* strains was isolated and used as

template for coupled transcription and translation *in vitro* (Figure 2). The pCPATE6

DNA was isolated from JM109 cells (lane 3 of Figure 2) or Top10[TM] cells (lanes 4-5 of

Figure 2). Total protein in the reaction mixtures was acetone-precipitated and equal

amounts were separated by SDS-PAGE (Figure 2A). The autoradiogram (Figure 2B)

indicated the presence of two [^{35}S- Met]-labeled protein products in lanes 3-5. The 46

kDa product likely represents the PATE preprotein while the 35 kDa product probably

represents a truncated protein product. There appears to be an in-frame internal initiation

codon in the coding region (Figure 1). Upon examination of the coding sequence, we

predict that an internal initiation codon occurs at nt 316 at an internal methionine codon,

corresponding to M106 (Figure 1). Upstream from the alternative start site there is a

Shine-Dalgarno consensus ribosome-binding sequence, GAGAAG (Shine and Dalgarno,

1974; Hunt, 1980). The smaller 35 kDa protein (Figure 2) likely represents transcription

and translation of this truncated polypeptide. It is unlikely that the smaller 35 kDa

protein represents a degradation product, since the addition of phenylmethylsulfonyl

fluoride (PMSF) did not alter the accumulation of the 46 kDa protein (lane 5 of Figure 2),

but did result in an increase in the amount of the [^{35}S-Met] labeled 35 kDa polypeptide.

In the absence of DNA template (lane 1), there was no [^{35}S-Met] labeling of any

endogenous *E. coli* proteins. As a negative control, the cotton *FatB* cDNA fragment was

ligated into the plasmid vector pGEM-7Zf(+) in reverse orientation relative to the *lacZ*

promoter and the subclone was designated pGEMPATE6. As shown in lane 2 of Figure
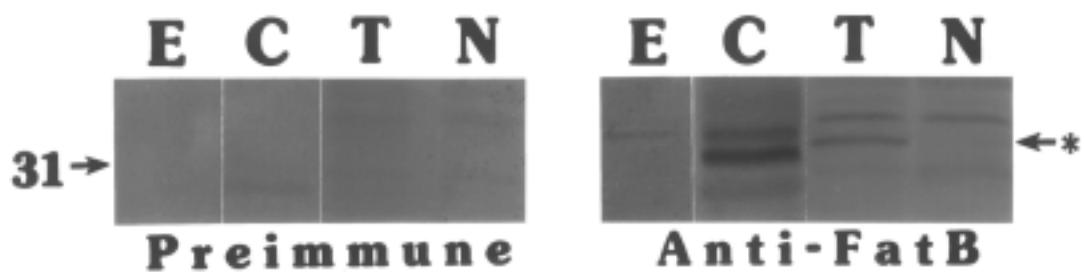
Figure 3: Detection of the endogenous cotton FatB in 30 dpa embryos (E) and 48 h dark-grown cotyledons (C), and expression of the cotton FatB in transformed *fad*D88 *E. coli* mutant lysates (T) (compared to nontransformed (N) *E. coli* lysates). Immunoblots were probed with 1:5,000 preimmune serum and 1:5,000 anti-FatB antibodies. The asterisk represents the 34.7 kDa antigen in transformed *E. coli* mutant lysate. The antigen detected in cotton homogenates are slightly larger, about 36.7 kDa. The co-migration of a 31 kDa standard protein is indicated by an arrow on the left.

2, no expression of the *PATE* coding region in pGEMPATE6 was observed, as would be expected.

Detection of FatB1 polypeptide

Polyclonal anti-peptide (RPKHAKSSANMDQITAKR; amino acid residues #395-412, Figure 1) antibodies were used to detect FatB proteins in crude cotton homogenates and *E. coli* lysates (Figure 3). In crude homogenates of 30dpa embryos and cotyledons of 48h dark-grown cotton seedlings, the anti-FatB antibodies recognized a 37 kDa protein, not observed in the blots probed with preimmune serum. This apparent molecular weight was estimated from plots of M_r in comparison with known protein molecular weight standards, and is consistent with a predicted molecular weight of the mature protein of 36.7 kDa (using Hitachi DNAsis software). The smaller molecular weight protein seen in the cotyledon lane probably represents a proteolytic degradation product, since its relative abundance differed from preparation to preparation or with extended freeze-thaw cycles.

In *E. coli* (*fad*D88 mutant) lysates, an immunoreactive 35 kDa protein was observed from cells transformed with pCPATE6 plasmid DNA but not in nontransformed cells. The size of this presumptive FatB protein product expressed in *E. coli* is consistent with the size of the major [^{35}S-Met]-labeled protein observed in *in vitro* transcription/translation experiments (Figure 2B). This supports the notion that indeed there is an internal initiation codon, which is preferred when the cotton cDNA clone is expressed in *E. coli*. For this reason, the anti-FatB antibodies recognized a smaller molecular weight protein in *E. coli* lysates (35 kDa) when compared to the FatB protein visualized in cotton embryo extracts or cotyledon extracts (37 kDa). The higher

68

molecular weight proteins seen in both the transformed and nontransformed lanes are probably *E. coli* proteins, recognized non-specifically by both preimmune and immune serum. As might be expected, no higher molecular weight proteins were observed in plant extracts, indicating most of the protein was processed to the mature form (data not shown).

Fatty acid release into the culture medium *in vivo*

*E. coli fadD*88 mutants lack acyl-CoA synthetase activity (Overath et al., 1969; Klein et al., 1970). When these *E. coli* cells are transformed with an acyl-ACP thioesterase, the free fatty acids produced in the cells cannot be esterified to CoA and are released into the medium (Dörmann et al., 1995). The truncated PATE polypeptide expressed in *E. coli*, lacks the complete stromal targeting domain (STD) but retains conserved catalytic residues (Figure 1). C347 would be required for the formation of the covalent thiol enzyme intermediate and H312 would be involved in general base catalysis, as described for plant acyl-ACP thioesterases (Yuan et al., 1996).

A four- to five-fold increase in 16:0 free fatty acid content was measured in the medium of transformed bacterial cells when compared with the nontransformed cells (equivalent number of cells and medium). In addition, although overall amounts were lower, there was a two- to three-fold increase in both 18:0 and 18:1 free fatty acids in the medium (Figure 4). These data indicate that the cotton *FatB* cDNA construct expressed in *E. coli* encodes a functional acyl-ACP thioesterase despite its apparent truncated size. In these *E. coli* cells, the double bond in 18:1 is in the delta-11 position ($18:1^{\triangle 11cis}$) (Dörmann et al., 1995). The 18:1 free fatty acids released by *E. coli* to the culture

medium is different from the major 18:1 fatty acids ($18:1^{\triangle 9cis}$) in higher plants (Voelker, 1996). Consequently, we examined acyl-ACP thioesterase activity toward $18:1^{\triangle 9cis}$-ACP substrate *in vitro* (below).

Acyl-ACP thioesterase activity assay

Acyl-ACP thioesterase activity assays were compared with palmitoyl-ACPs and oleoyl-ACPs ($18:1^{\triangle 9cis}$) as substrates. There was a clear preference for the palmitoyl-ACP over the oleoyl-ACP *in vitro* (Figure 5). This confirmed that the cotton cDNA encodes a functional acyl-ACP thioesterase, consistent with the free fatty acid release from the *fad*D88 *E. coli* mutants. Moreover, a preference for saturated acyl-ACPs (FatB) over unsaturated acyl-ACPs (FatA) is indicated and hence by functional criteria, the cotton cDNA would be classified as a FatB thioesterase. To examine whether our results were obscured by any potential background activity of *E. coli* acyltransferase, lysates of transformed as well as nontransformed *fad*D88 mutants were tested for thioesterase and acyltransferase activities (Figure 6). During the preparation of the lysates, both a soluble protein and a membrane-bound protein fractions were collected. The soluble fraction represents all proteins which were collected in the supernatant after cells were lysed, and there was about a ten-fold greater thioesterase activity (open bars) in transformed *fad*D88 mutants over the nontransformed mutants. The release of [^{14}C-1] palmitic acid was confirmed by TLC and radiometric scanning. In addition, acyltransferase activity was almost non-detectable in the soluble fraction. This was also confirmed by TLC
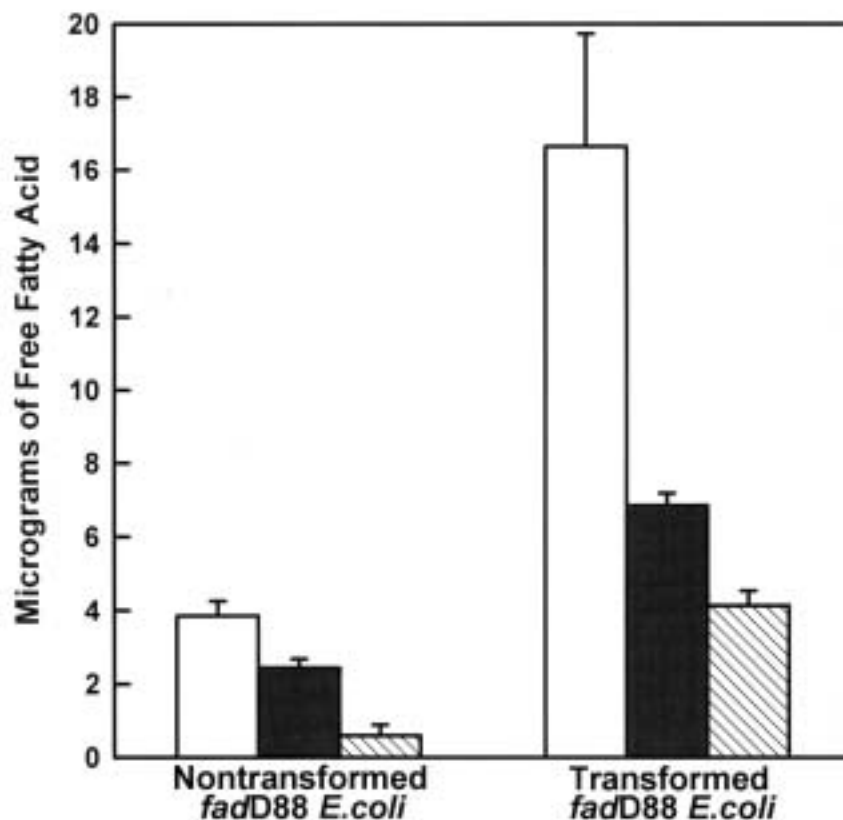
Figure 4: Release of free fatty acids into the culture medium of *E. coli fadD*88 cells

containing pCPATE6 plasmid. Fatty acid methyl esters (FAMEs) of palmitic acid (open

bars), stearic acid (solid bars), and oleic acid (hatched bars) were analyzed by GC-FID.

Error bars represent the standard deviation of data obtained from four independent

experiments.
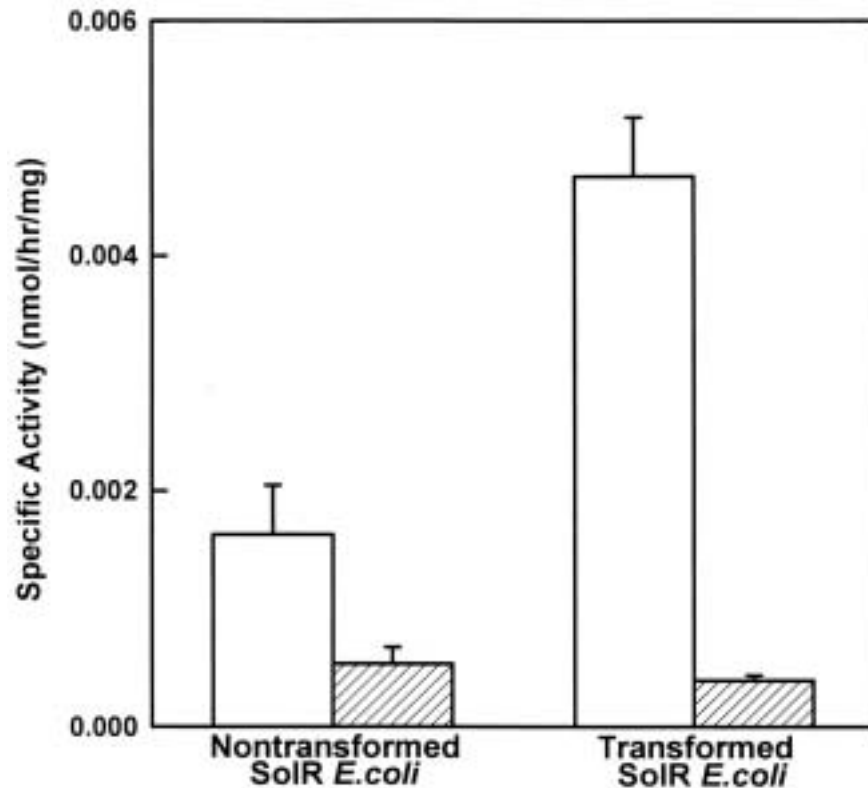
Figure 5: Thioesterase activities in lysates of transformed *E. coli* SolR cells expressing the cotton *PATE* cDNA SKCPc115b as a *lac*Z-PATE fusion polypeptide compared with thioesterases of lysates of nontransformed *E. coli* SolR cells. The thioesterase specific activities were determined for hydrolysis of [^{14}C]-palmitoyl-ACP (open bars) and for [^{14}C]-oleoyl-ACP (hatched bars). The values shown depict the mean and range from two independent determinations.
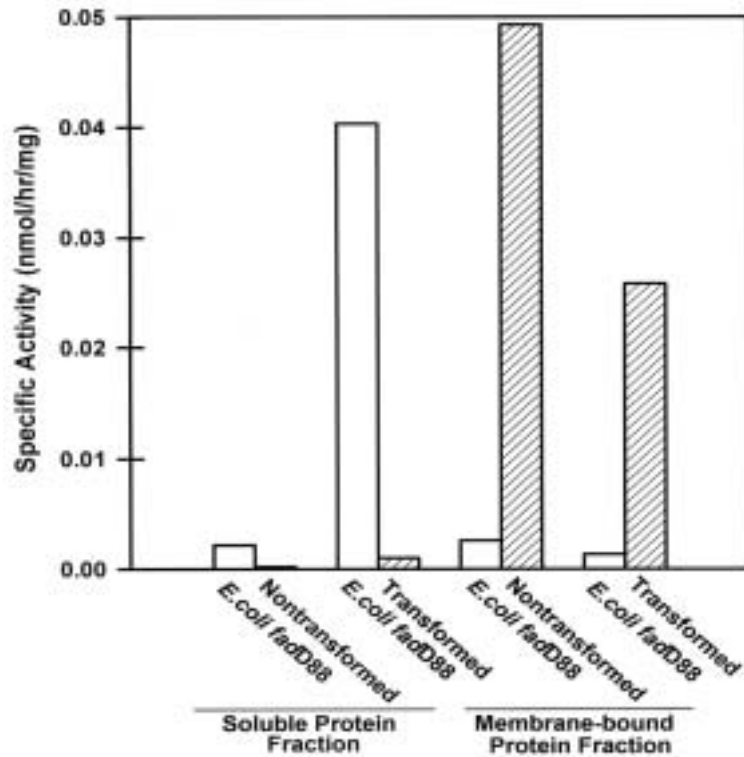
Figure 6: Specific activity of thioesterase activity (open bars) compared to acyltransferase activity (hatched bars) toward [^{14}C]palmitoyl-ACP. Activities were assayed in the soluble lysate fraction and wash (pellet dissolved with lysoszyme) fractions of *E. coli fad*D88 cells transformed with the pCPATE6 plasmid compared to nontransformed *E. coli fad*D88 cells.

since no radioactivity was detected in the polar lipids (data not shown).

Insoluble pellets from the initial lysis were incubated with lysozyme, which released most of the remaining membrane-bound proteins including acyltransferase. No significant thioesterase activity was recovered, but by contrast, about 100 times more acyltransferase activity (hatched bars) was detected in the membrane protein fraction when compared with the soluble protein fraction as predicted. These data demonstrated that the thioesterase activity is indeed in the soluble lysate and is clearly distiguishable from the endogenous acyl-ACP acyltransferase activity.

Discussion

Previously, we identified a cotton *FatB* cDNA clone (Pirtle et al., 1999) with substantial sequence identity with the *Arabidopsis FatB*, palmitoyl-ACP thioesterase cDNA (Dörmann et al., 1995). In attempts to demonstrate that the cDNA encodes a functional enzyme, we have expressed this protein in various *E. coli* strains. Upon expression of the cotton *FatB* cDNA in *E. coli*, a truncated recombinant protein was detected. In *in vitro* transcription/translation experiments, a 35 kDa [^{35}S-Met]-labeled protein product was detected by autoradiography (Figure 2B). This truncated 35 kDa protein product was also recognized by polyclonal anti-peptide antibodies on immunoblots of *E. coli* cell lysates (Figure 3). The processed, mature form of the cotton FatB protein is estimated to be about 37 kDa, which was immunologically cross-reactive in cotton embryo and cotyledon extracts. Thus, there appears to be an alternative translational start site (Figure 1) within the cDNA coding region that seems preferentially

used when the cDNA is expressed in *E. coli*. Translation initiation at an internal methionine, M106, would result in a truncated protein, which lacks the stromal targeting domain (STD). The insertion of the STD into *E. coli* membranes may cause the *E. coli* cells to overaccumulate inclusion bodies and become unstable. Therefore, the lack of a STD in the truncated recombinant protein may have a beneficial role in reducing the toxicity of these proteins in *E. coli*.

We have examined the *in vivo* and *in vitro* thioesterase activity of the cotton *FatB* cDNA. When *E. coli fadD*88 mutants were transformed with the cotton *FatB* cDNA, a significant increase in 16:0 free fatty acid content was measured in the culture medium (Figure 4). Although overall amounts were lower, there was also a slight increase in 18:0 and 18:1 free fatty acids in the medium. However, the 18:1 free fatty acids in *E. coli* (18:1 ^{11cis}) differ in structure from the major 18:1 fatty acids in higher plants (18:1 ^{9cis}). Thus, to further demonstrate that the cotton cDNA encodes a FatB rather than a FatA acyl-ACP thioesterase, activity assays were compared with palmitoyl-ACPs and oleoyl-ACPs (18:1 ^{9cis}). There was a clear preference for palmitoyl-ACPs over oleoyl-ACPs (Figure 5). These data are consistent with the *in vivo* results, supporting the notion that the cotton *FatB* cDNA expressed in *E. coli* encodes a functional acyl-ACP thioesterase, despite its truncated size.

In addition, we have confirmed that the thioesterase activity in transformed *E. coli* cell lysates was not obscured by endogenous *E. coli* acyltransferase activity (Figure 6). We were able to easily discern thioesterase activity from endogenous acyl-ACP acyltransferase activity by assaying different fractions of the cell lysate preparations, and

the majority of the thioesterase activity was located in the soluble protein fraction, not the membrane-bound protein fraction. We conclude that the increase in acyl-ACP hydrolysis and the increase in free fatty acid production in transformed *E. coli* cells is attributed to the cotton *FatB* cDNA, which encodes a acyl-ACP thioesterase.

Browse and Sommerville (1991) originally hypothesized that there is a second plant acyl-ACP thioesterase other than the oleoyl-ACP thioesterase. Currently, it is widely accepted that there are two major types of acyl-ACP thioesterases in plants (Somerville et al, 2000; Ohlrogge et al, 1997). The major class of thioesterases are designated as FatA, which are most active towards $18:1^{\Delta 9}$-ACP substrates. The second class of thioesterases, designated FatB, are typically active towards 16:0-ACP substrates. These two acyl-ACP thioesterases are found in most tissues of all plant species (Somerville et al., 2000). This supports the notion that fatty acid chain elongation usually ends at 16:0 or 18:0. However, a few plant species such as coconut, many species of *Cuphea*, and California bay (*Umbellularia californica*) have thioesterases that yield shorter-chain (C10-C14) saturated fatty acids. Because these thioesterases prefer saturated acyl-ACPs, they are also classified as FatB thioesterases. These enzymes prematurely terminate fatty acid biosynthesis resulting in the incorporation of C10, C12, and C14 fatty acids into seed triacylglycerols. Unlike the ubiquitous FatB palmitoyl-ACP thioesterase, the shorter-chain acyl-ACP thioesterases are species-specific and found only in seeds.

One *Arabidopsis FatB* cDNA clone encoding a long-chain acyl-ACP thioesterase has been analyzed (Dörmann et al., 1995; Voelker, 1996). When expressed in *E. coli*,

the enzyme product had the highest activity toward 16:0-ACP, but still significant

activities for other long acyl-ACPs such as 18:0-ACP and 18:1-ACP. Furthermore,

Dörmann et al. (1995) reported that the activity pattern of this enzyme may overlap with

that of the FatA, oleoyl-ACP thioesterase (OTE) in *Arabidopsis* such that both enzymes

show thioesterase activity toward 16:0-ACP and 18:1-ACP (18:1$^{\Delta 9cis}$). Thus it is possible

that the highly active OTE in *Arabidopsis* obscures the activity of the FatB thioesterase,

making it difficult to detect FatB acyl-ACP thioesterase activity in plant extracts. In

contrast, a FatB-like activity predominated in some cotton tissues such as cotyledons of

cotton seedlings and 30dpa developing embryos (Pirtle et al., 1999). Based on these

activities in crude cotton homogenates and similarities of the predicted amino acid

sequence to the *Arabidopsis* FatB thioesterase, the cotton enzyme product was also

termed a FatB thioesterase (Pirtle et al., 1999). However, thioesterase activity assays of

E. coli cell lysates reveal that, unlike the broad substrate specificity of the *Arabidopsis*

FatB thioesterase with similar activities toward both 16:0-ACPs and 18:1-ACPs, the

cotton FatB thioesterase is more specific for palmitoyl-ACP. Dörmann et al. also

suggested that this second thioesterase activity for acyl-ACPs other than oleoyl-ACP can

be easily detected in some plants such as California Bay, *Cuphea*, coconut, and palm

mesocarp, while it may be down regulated in other plants as seen in *Arabidopsis* and

Brassica. Cotton may be included in the group of plants in which the activities of the

FatA and FatB thioesterases are easily distingushable. Thus in cotton, the specific

activities of the FatB thioesterase may predominate over the FatA thioesterase in some

tissues, suggesting that in these tissues the demand for extraplastidial, saturated fatty

acids may be higher. Collectively, our results indicate that the cotton *FatB* cDNA

isolated encodes a functional thioesterase with a preference *in vivo* and *in vitro* for

saturated acyl-ACPs (FatB) over unsaturated acyl-ACPs (FatA). The availability of this

functional, full-length clone will allow us to further evaluate the role of this enzyme in

saturated lipid synthesis in transgenic cotton plants.

References

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

Chapman KD, Sprinkle WB (1996) Developmental, tissue-specific, and environmental factors regulate the biosynthesis of *N*-acylphosphatidylethanolamine in cotton (*Gossypium hirsutum* L.), *J. Plant Physiol.* 149: 277-284.

Christie WW (1982) Lipid Analysis, 2nd edition, Pergamon Press, New York, pp.52-54.

Cline K, Henry R (1996) Import and routing of nucleus-encoded chloroplast proteins, *Annu. Rev. Cell Dev. Biol.* 12: 1-26.

Dehesh K, Edwards P, Hayes T, Cranmer AM, Fillatti J (1996) Two novel thioesterases are key determinants of the bimodal distribution of acyl chain length of *Cuphea palustris* seed oil, *Plant Physiol.* 100: 203-210.

Dörmann P, Voelker TA, Ohlrogge JB (1995) Cloning and expression in *Escherichia coli* of a novel thioesterase from *Arabidopsis thaliana* specific for long-chain acyl-acyl carrier proteins, *Arch. Biochem. Biophys.* 316: 612-618.

Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides, *FEBS Lett.* 261: 455-458.

Harwood JL (1988) Fatty acid metabolism, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39: 101-138.

Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids, *Biochim. Biophys. Acta* 1301: 7-56.

Hunt T (1980) The initiation of protein synthesis, *Trends Biochem. Sci.* 5: 178.

Jones A, Davies HM, Voelker TA (1995) Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases, *Plant Cell* 7: 359-371.

Kinney AJ (1997) Genetic engineering of oilseeds for desired traits, *In*: Setlow J.K. (ed.) Genetic Engineering, vol. 19, Plenum Press, New York, pp. 149-166.

Klein K, Steinberg R, Fiethen B, Overath P (1971) Fatty acid degradation in *Escherichia coli*: An inducible system for the uptake of fatty acids and further characterization of *old* mutants, *European J. Biochem.* 19: 442-450.

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

McCarthy AD, Hardie DG (1984) Fatty acid synthase - an example of protein evolution by gene fusion, *Trends Biochem. Sci.* 9: 60-63.

Ohlrogge JB (1982) Fatty acid synthetase: plants and bacteria have similar organization, *Trends Biochem. Sci.* 7: 386-387.

Ohlrogge JB, Browse J (1995) Lipid biosynthesis, *Plant Cell* 7: 957-970.

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 109-136.

Ohlrogge JB, Jaworski JG, Post-Beittenmiller D (1993) *De novo* fatty acid biosynthesis, *In*: Moore T.S. (ed.) Lipid metabolism in plants, CRC Press, Florida, pp.3-32.

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

Overath P, Pauli G, Schairer HU (1969) Fatty acid degradation in *Escherichia coli*: An inducible acyl-CoA synthetase, the mapping of *old*-mutations, and the isolation of regulatory mutants, *European J. Biochem.* 7: 559-574.

Pappan K, Austin-Brown S, Chapman KD, Wang X (1998) Substrate selectivities and lipid modulation of plant phospholipase Dα, -β, and -γ, *Arch. Biochem. Biophys.* 353: 131-140.

Pirtle RM, Yoder DW, Huynh TT, Nampaisansuk M, Pirtle IL, Chapman KD (1999) Characterization of a palmitoyl-acyl carrier protein thioesterase (FatB1) in cotton, *Plant Cell Physiol.* 40: 155-163.

Schãgger H (1994) Denaturing electrophoretic techniques, *In*: Von Jagow G., Schãgger H. (eds.), A Practical Guide to Membrane Protein Purification, Academic Press, San Diego, pp. 59-79

Shanklin J (2000) Overexpression and purification of the *Escherichia coli* inner membrane enzyme acyl-acyl carrier protein synthase in an active form, *Protein Expression and Purification* 18: 355-360.

Shine J, Dalgarno L (1974) The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosomal binding sites, *Proc. Natl. Acad. Sci.USA* 71: 1341-1346.

Somerville C, Browse J, Jaworski JG, Ohlrogge JB (2000) Lipids, *In*: Buchanan BB,

Gruissem W, Jones RL (eds.) Biochemisrty and Molecular Biology of Plants, Courier Companies, Inc., Rockville, pp.456-527.

Speicher DW (1999) Electrophoresis, *In*: Coligan JE, Dunn BM, Ploegh HL, Speicher DE, Wingfield PT (eds.) *Current Protocols in Protein Science*, vol.1, John Wiley & Sons, Inc., Seattle, pp. 10.1.1-10.1.34.

Voelker T (1996) Plant acyl- ACP thioesterases: chain-length determining enzymes in plant fatty acid biosynthesis, *Genetic Engineering* 18: 111-113

Voelker TA, Davies HM (1994) Alteration of the specificity and regulation of fatty acid synthesis of *E. coli* by expression of a plant medium-chain acyl-ACP thioesterase, *J. Bacteriol.* 176: 7320-7327.

Yoder DW, Nampaisansuk M, Pirtle IL, Chapman KD, Pirtle RM (1999) Molecular cloning and nucleotide sequence of a gene encoding a cotton palmitoyl-acyl carrier protein thioesterase, *Biochim. Biophys. Acta* 1446: 403-413.

Yuan L, Nelson BA, Caryl G (1996) The catalytic cysteine and histidine in the plant acyl-acyl carrier protein thioesterases, *J. Biol. Chem.* 271: 3417-3419.

CHAPTER 3

DEVELOPMENT AND CHARACTERIZATION OF TRANSGENIC COTTON PLANTS HARBORING SENSE AND ANTISENSE COTTON FATB EXPRESSION CONSTRUCTS

Abstract

A palmitoyl-acyl carrier protein (ACP) thioesterase (FatB) from cotton (*Gossypium hirsutum* L.) was previously characterized and shown to have *in vitro* hydrolytic activity toward palmitoyl-ACP and much less toward oleoyl-ACP, with the highest specific activity in extracts from 30 days post anthesis (dpa) embryos (Pirtle et al, 1999). The relatively high level of palmitic acid (22 mol%) in cottonseeds may be due in part to the activity of this FatB thioesterase. In this study, we generated transgenic cotton plants harboring sense and antisense *FatB* expression constructs under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S constitutively active promoter. Over-expression of the *FatB* cDNA resulted in elevated levels of palmitic acid (up to 70% of total) in somatic embryos compared to vector-only (pBI 121) control somatic embryos and compared to various stages of zygotic embryos, which have only about 20-25% palmitic acid throughout development. Immunoblots revealed substantially higher amounts of the immuno-reactive 37kDa FatB protein in cell-free extracts of somatic

embryos in these transgenic lines compared to controls. Expression of the *FatB* cDNA in antisense orientation did not show a reduction in the relative percentage of palmitic acid in somatic embryos. In addition, the fatty acid composition of pooled T_1 seeds, cotyledons of 2d-old cotton seedlings, and mature leaves were similar to wild-type (cv. Coker 312) and vector-only (pBI 121) control transgenic plants. However, about 30% of the "anti-sense" transgenic plants displayed a dwarfed phenotype, while the others had a phenotype similar to wild-type. These dwarfed plants had significantly smaller mature leaves all with smaller cells including smaller stomata and guard cells suggesting that these plants may have less palmitic acid available for incorporation into extraplastidial membrane lipids during cell expansion. PCR amplification of the *FatB* cDNA confirmed that the transgene was incorporated into the genome of the dwarfed as well as the non-dwarfed anti-sensed plants. These data collectively revealed that the manipulation of FatB thioesterase activity altered palmitic acid content in cotton indicating that FatB thioesterase plays a direct role in regulating palmitic acid levels in this plant system.

Abbreviations

ACP, acyl-carrier protein; bp, base pair(s); dpa, days post anthesis; GC-FID, gas chromatography - flame ionization detection; PATE, palmitoyl-acyl carrier protein thioesterase (also to designate transgenic plants harboring the overexpression construct; e.g. PATE plantlets); ASPATE, anti-sense PATE (to designate transgenic plants harboring the anti-sense PATE construct); PCR, polymerase chain reaction; TAG(s), triacylglycerol(s).

Introduction

Lipids have diverse roles in plants and are essential for normal growth and development. One important role of plant lipids, as in other organisms, is as structural components of cellular membranes (Ohlrogge and Browse, 1995; Harwood, 1996; Ohlrogge and Jaworski, 1997). Studies with *Arabidopsis* mutants have shown that the complement of lipid types is important for the functional integrity of membranes (Browse et al., 1986; Hugly and Somerville, 1992; Miquel et al., 1993; Wu et al., 1997). Photosynthetic efficiency is greatly reduced in *Arabidopsis fad*2 *fad*6 mutants, which lack diunsaturated fatty acids $18:2^{\Delta 9,12}$ and $16:2^{\Delta 7,10}$ (Somerville et al., 2000). In these mutants, triunsaturated derivatives, $18:3^{\Delta 9,12,15}$ and $16:3^{\Delta 7,10,13}$, are reduced as well affecting the integrity of the thylakoid membranes which consist normally of about 70% polyunsaturated fatty acids. Membrane lipid compositions also were shown to influence plant responses to freezing (Havaux, 1987; Hugly and and Somerville, 1992; Wu et al., 1997; Somerville et al., 2000). Recently, the *Arabidopsis* triple mutant *fad*3-2 *fad* 7-2 *fad*8, completely deficient in 16:3 and 18:3 fatty acids, was used to demonstrate that trienoic fatty acids are required to maintain chloroplast function at low temperatures (Routaboul et al., 2000).

Lipids also are an important energy reserve in plants (Harwood, 1996; Somerville et al., 2000). Lipids stored in seeds are usually in the form of triacylglycerols (TAGs). Mobilization of these storage lipids is essential to provide carbon and chemical energy for seed germination and seedling growth (Trelease and Doman, 1984; Somerville et al., 2000). Plant seed oils have an additional nutritional importance

because 18:2 polyunsaturated fatty acids are essential to the human diet (Harwood, 1996). Recently it has been shown that genetic manipulation to alter the fatty acid composition of storage TAGs is possible without detrimental effects to the host plant, thus resulting in the production of novel oils with improved nutritional characteristics (Kinney, 1994; Harwood, 1996; Kinney, 1997; Kinney and Knowlton, 1998). The focus of these metabolic engineering projects is to target some enzymes of the fatty acid biosynthetic pathway and to examine how they regulate plant lipid accumulation.

Higher plants synthesize fatty acids *de novo* in the stromal compartment of plastids with a type II, fully dissociable fatty acid synthase complex (Ohlrogge and Browse, 1995; Harwood, 1996; Kinney, 1997; Ohlrogge and Jaworski, 1997). Acyl chains esterified to an acyl carrier protein (ACP) undergo chain elongation by the sequential addition of two-carbon units from malonyl-ACP. Hydrolysis of the acyl-ACP thioester bond by an acyl-ACP thioesterase enzyme terminates acyl chain elongation. The role of these thioesterases in the regulation of *de novo* fatty acid synthesis in plants has been examined in many studies during the past decade (Browse and Somerville, 1991; Ohlrogge et al., 1993; Harwood, 1996; Somerville et al., 2000). In particular, some researchers have focused on investigating the mechanisms for determining fatty acid chain length (Dörmann et al., 1994; Dörmann et al., 1995; Eccleston et al., 1996; Voelker, 1996). Currently, it is believed that the substrate specificities of the acyl-ACP thioesterases play a major role in determining when the acyl chains are cleaved and released from the ACPs, and thus directly influence the pools of free fatty acids available for export to extraplastidic compartments. Typically fatty acid chain elongation is

terminated at C16:0 or C18:1 resulting in the production of 16:0-ACP and 18:1-ACP as the major products of plastid fatty acid biosynthesis. Two principal types of acyl-ACP thioesterase exist in plants. The FatA class hydrolyzes specifically $18:1^{\Delta 9}$-ACP while the FatB class is most active with saturated acyl-ACPs. The FatA $18:1^{\Delta 9}$-ACP thioesterase and the FatB 16:0-ACP thioesterase are found in most tissues of all plant species (Somerville et al., 2000). However, some FatB thioesterase isoforms are found only in seeds and are species-specific. Plants such as California bay (*Umbellularia californica*) and many species in the genus, *Cuphea*, have FatB thioesterases that are active toward medium chain acyl-ACPs (C10:0-C14:0) and thus prematurely terminate fatty acyl-chain elongation before they are C16:0 or C18:0 long. Consequently these plants accumulate medium-chain fatty acids, in their seed TAGs (Voelker et al, 1992; Dörmann et al., 1993; Dehesh et al., 1996).

The DNA sequences of the medium-chain-specific thioesterases were Dörmann used to identify a FatB thioesterase in *Arabidopsis* (AtFATB1; Dörmann et al., 1995; et al, 2000). An expressed sequenced tag (est) from *Arabidopsis* was found with a cDNA sequence that was similar to the medium-chain thioesterases. *Arabidopsis* plants do not accumulate medium-chain fatty acids in their tissues. Expression of the *AtFATB1* thioesterase in *Escherichia coli* revealed that this cDNA encoded an enzyme most active *in vitro* toward 16:0-ACP, with some overlapping activity for $18:1^{\Delta 9}$-ACP, 18:0-ACP, and 14:0-ACP (Dörmann et al, 1995). Although the previously discovered FatB thioesterases were seed-specific, the AtFATB1 was found in leaves, roots, and siliques. The *Arabidopsis* cDNA clone encoded the first FatB long-chain acyl-ACP thioesterase.

We recently isolated and characterized a palmitoyl-ACP thioesterase (FatB) in cotton that has 63% identity in amino acid sequence to the *Arabidopsis* FatB long-chain acyl-ACP thioesterase (Chapter 1; Pirtle et al, 1999; Yoder et al, 1999). In zygotic embryo extracts, the palmitoyl-ACP thioesterase activity was highest at the maximum rate of reserve accumulation (oil and protein), occurring about 30-35 days post anthesis (dpa). When expressed in *E. coli*, the cotton FatB thioesterase was shown to have hydrolytic activity *in vitro* toward 16:0-ACP with much less toward $18{:}1^{\Delta 9}$-ACP (Chapter 2). Data from the previous two chapters provided evidence that an *Arabidopsis* FatB acyl-ACP thioesterase homologue was present in cotton and I hypothesized that this enzyme was responsible in part for the high levels of palmitic acid incorporated into cottonseed oil. In this chapter, I described the generation of transgenic cotton plants harboring sense and antisense *FatB* expression constructs under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S strong, constitutively active promoter and examined the *in planta* consequences of altered FatB expression. Manipulation of the FatB thioesterase expression directly influenced palmitic acid levels. These transgenic cotton plants should provide new insights into the influence of palmitic acid content on plant growth and development.

Material and Methods

Transformation Vectors

Binary vectors, designated pPATE and pASPATE, were constructed by Mongkol Nampaisansuk in Dr. R. Pirtle's Laboratory for use in cotton transformations (Figure 1;
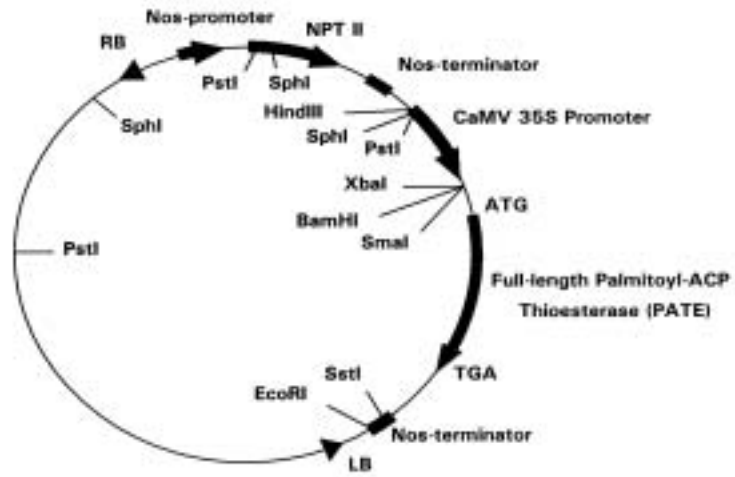
A. PATE Sense Construct
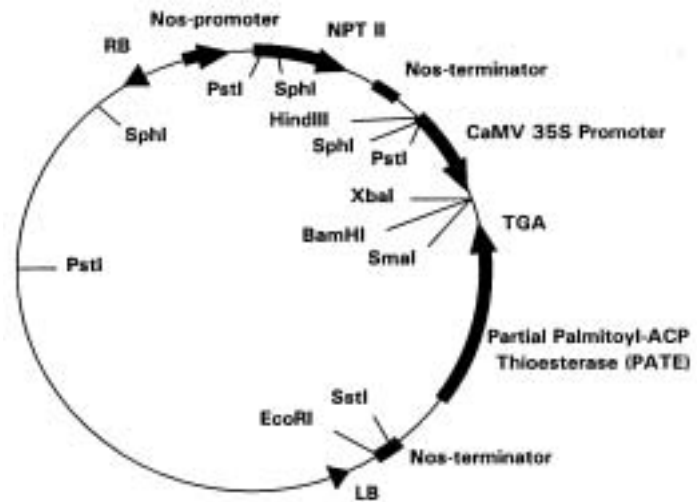
B. PATE Antisense Construct

Figure 1. Expression constructs used in transformation experiments (Conducted by

Mongkol Nampaisansuk in Dr. R. Pirtle's Laboratory). (A) Sense expression construct

includes the full-length palmitoyl-ACP thioesterase (PATE) cDNA sequence inserted in

sense orientation downstream from the Cauliflower Mosaic Virus (CaMV) 35S promoter.

(B) Anti-sense expression construct includes the partial palmitoyl-ACP thioesterase

(PATE) cDNA sequence inserted in anti-sense orientation downstream from the CaMV

35S promoter.

The pPATE vector was constructed with a RT-PCR product generated and sequenced by Tu Huynh in Dr. K. Chapman's Laboratory). This vector contains the Ti-plasmid left and right border (LB, RB) inverted repeat sequences for integration into the cotton genomic DNA. The T-DNA segment harbors the selectable marker, nptII, regulated by the nos (nopaline synthesis) promoter. The palmitoyl-ACP thioesterase (PATE) cDNA sequence was introduced in sense or anti-sense orientation between unique *Xba*I and *Sst*I sites, downstream from the CaMV 35S promoter into the pBI 121 vector in the place of β-glucuronidase. The binary vectors were introduced into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation and maintained with kanamycin selection conferred by nptII expression. For plant transformation / plant regeneration controls, transgenic cotton plants were produced using the parent binary vector, pBI 121 (Clonetech; parent vector of pPATE and pASPATE) with the same *Agrobacterium* strain.

Cotton Transformation

Cotyledon pieces (~3 mm^2) were excised from 7 to14-d-old cotton (*Gossypium hirsutum* L., cv. Coker 312) seedlings (germinated aseptically according to Thomas et al., 1995 and Trolinder and Goodin, 1998, but grown at 30°C under 14 h photoperiod, 60:mol/s/m^2) and co-cultivated with 6X10^8 cells/mL *Agrobacterium tumefaciens* (strain LBA4404) harboring the binary vector pBI 121 for vector-only control experiments, or pPATE and pASPATE for fatty acid modification. *Agrobacterium*-mediated transformation (conducted by Shea Brown) and plant regeneration were done according to Firoozabay et al. (1987), Umbeck et al. (1987), and Thomas et al. (1995) with minor modifications. Briefly, cotton cotyledon explants were placed in co-cultivation medium

(MS medium, 1.5% w/v sucrose, 40 μM acetosyringone, 2 mM MES-NaOH, pH 5.5) along with an equal volume of *Agrobacterium* cell suspension. This mixture was placed under vacuum (25" Hg) for 8 min, and equilibrated to 25°C for an additional 75 min. Explants were blotted on sterile filter paper and placed on G1 medium (MS salts with 3% w/v glucose) for 3 d at 25°C (Thomas et al., 1995). Explants were transferred to G2 medium (MS medium, 100 mg/L inositol, 1 μM thiamine, 25 μM 6-(g-g-dimethylallylaminopurine) (2ip), 0.5 μM napthaleneacetic acid (NAA), 3% w/v glucose, pH 5.8, 0.2% w/v Phytagel (Sigma) supplemented with 400 mg/L carbenicillin and 50 mg/L kanamycin) to induce calli formation (Thomas et al., 1995). The transformed calli were subcultured to fresh G2 medium every 2-4 weeks. After 3-6 months, proliferating transgenic calli were transferred to a modified MSOB medium (modified from Thomas et al., 1995 to contain MS salts, B-5 vitamins, 1.9 mM potassium nitrate, 100 mg/L inositol, 3% w/v glucose, pH 5.8, with 0.2% Phytagel) supplemented with 200 mg/L carbenicillin and 50 mg/L kanamycin). Developing embryos were recovered after 6-16 weeks and placed on MSOB medium without antibiotics. Elongated embryos were transferred to MS3 medium (MS salts, 0.4 μM thiamine-HCl, 0.5 μM pyridoxine-HCl, 0.8 μM nicotinic acid, 1% w/v glucose, pH 5.8 with 0.8 g/L Phytagel and 4 g/L agar) for root formation. Small plantlets were transferred to soil, hardened off, then transferred to greenhouse conditions (14 h photoperiods, supplemented with high intensity Na- and Hg-vapor lamps when necessary, 35°C day, 25°C night) for production of flowers and bolls. Plants were fertilized biweekly with a dilute solution of Miracle Gro^{TM}, and flower production was

stimulated with SuperBloom^{TM} (when necessary). Flowers were selfed, tagged at anthesis, and the progress of boll development was monitored daily.

Lipid Extraction, Preparation of Fatty Acid Methyl Esters (FAMEs), and Gas Chromatography

Equivalent amounts (protocol for 0.4 g) of cotton were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Hot isopropanol (70°C, 1 mL) was added to the sample and the mixture was incubated at 70°C for 30 min. Chloroform (0.5 mL) was added to the mixture and samples were placed at 4°C for an overnight extraction. The mixture was partitioned into two layers by the addition of chloroform (0.5 mL) and 1 M KCl (1 mL). The organic layer (bottom layer) was washed three additional times with 1 M KCl (2 mL). The organic layer was dried under nitrogen and the lipid residue was resuspended in tetrahydrofuran (THF, 0.5 mL). The lipids were transesterified in acidic methanol (1% sulfuric acid in methanol, 1 mL) according to Christie (1982). Briefly, the lipid residue in THF and acidic methanol was refluxed at 65-70 ^{o}C for 30 min. The methyl esters were recovered in hexane (1 mL of 5% NaCl and 2 mL hexane), washed with potassium bicarbonate (2% KHCO$_3$, 2 mL) and dried over sodium sulphate columns. FAMEs were analyzed by gas chromatography (GC) and quantified by flame ionization detection (FID) as described by Chapman and Trelease (1991). GC-FID analyses were conducted on a Hewlett-Packard 5890 Series II Gas Chromatograph using a 30 M DB-23 capillary column (0.25 mm i.d., 0.25 μm film thickness) and isothermal elution at 200°C (both injector and detector set at 250°C) with N$_2$ as the carrier gas.

Protein Extraction and Immunoblotting

Protein was extracted from various cotton tissues analyzed according to Fergusen et al. (1996). Briefly, cotton tissues of equivalent fresh weights were homogenized with a motorized pestle in extraction buffer (0.3 mL, 0.5 M Tris, pH 8.65, 50 mM EDTA, 100 mM KCl, 2% ß-mercaptoethanol) and saturated phenol (0.3 mL, phenol saturated with 1 M Tris, pH 7.9). The homogenates were centrifuged at 13,000 Xg for 15min and the phenol phase (bottom phase) was washed two times with equal volumes of the extraction buffer. The phenol layer was diluted to 1.5 mL with 0.1 M ammonium acetate (in methanol) and placed at -20°C overnight. The precipitated protein was pelleted at 13,000 Xg and washed two times with 0.1 M ammonium acetate and one time with cold (-20°C) acetone. The protein pellet was resuspended in extraction buffer for protein determination according to Bradford (1976) and boiled in SDS treatment buffer for western blotting as described in Chapter 2. The protein samples were separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane for immuno-detection as described in Chapter 2.

Cell Preparation for Light Microscopy

Young and mature leaves from transgenic and control plants were harvested and placed on ice. Leaves were de-veined and placed into 95% ethanol for the removal of chlorophyll overnight at 4°C. Leaves were re-hydrated with water for about 1h prior to staining. An epidermal layer of cells was isolated with forceps from the adaxial surface of the leaves. The layer of cells was fixed onto microscope slides with 95% ethanol and stained with a dilute solution of eosin (5% (v/v) in distilled water; cytoplasm-staining

dye). Cells were viewed at 250X magnification with a Zeiss Axiophot (Germany) light

microscope and images were photographed with an attached camera (Sony Hyper HAD

color video camera; Dr. Gross' Laboratory), for measurements.

DNA isolation and Polymerase Chain Reaction

DNA from young leaves of transgenic and control plants were extracted

according to Paterson et al. (1993). Briefly, up to 4 g of leaves were homogenized in a

minichop (SEB) blender (2X 20 sec) with DNA extraction buffer (20 mL ice-cold, 0.35

M glucose, 0.1 M Tris-HCl, pH 8.0, 0.005 M Na_2-EDTA, pH 8.0, 2% (w/v)

polyvinylpyrolidone (PVP 40), 0.1% (w/v) diethyldithiocarbamic acid (DIECA), 0.1%

(w/v) ascorbic acid, 0.2% (w/v) mercaptoethanol, solution adjusted to pH 7.5 before use).

A nuclei pellet was obtained after centrifugation at 2700 Xg (20 min at 4°C). Nuclei lysis

buffer (8 mL, 0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 0.02 M Na_2-EDTA, pH 8.0, 2% (w/v)

hexadecyltrimethylammonium bromide (CTAB), 2% (w/v) PVP40, 0.1% (w/v) DIECA,

1% (w/v) ascorbic acid, 0.2% (w/v) mercaptoethanol) was added to the pellet and the

mixture was incubated at 65°C for 30 min. Chloroform-isoamyl (CIA) alcohol (10 mL,

24:1) was added to each sample to remove proteins. After 2-3 CIA washes, DNA was

precipitated from the final CIA supernatant with isopropanol (0.6 volumes). The DNA

aggregate was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 65°C for

10-30 min and the purity of the DNA sample was determined spectrophotometrically at

260 nm and 280 nm (purity of DNA was judged suitable when the 260 nm:280 nm ratio

was >2:1; 1 O.D.=50μg of DNA).

Nuclear genomic DNA samples (300 ng) were used as templates in PCR reactions with *PfuTurbo* DNA polymerase (Stratagene) and forward PATE amplimer (5'-GGGTGATGTTGTTCAAGTCG-3') and reverse PATE amplimer (5'-TCAAGGATCCAGCC-3'; primers synthesised at Biosynthesis, Inc. and supplied by Dr. Pirtle) according to manufacturer's protocols to amplify the appropriate fragment of 354 bp from the transgene (*FatB* cDNA) and 715 bp from the endogenous *FatB* gene (See Figure 10A). PCR conditions (Perkin Elmer 2400 thermal cycler) were as follows. There was an initial denaturation of DNA fragments at 95°C for 5 min. Amplification of the targeted region was achieved through 30 cycles of 95°C for 45 sec, 58°C for 45 sec, and 72°C for 2 min, followed by a final polymerization step at 72°C for 7 min. The 354 bp and 715 bp PCR products were separated on 2% agarose gels and the bands visualized following ethidium bromide staining (Sambrook et al., 1989).

Results

Production of Transgenic Plants

Selected stages of transformation, tissue culturing, and plant regeneration are illustrated in Figure 2. Transgenic calli tissues arising from the cotton cotyledon explants after cocultivation with *Agrobacterium* harboring the expression constructs (Figure 1) were the first tissues available for examination of a change in fatty acid composition. The calli tissues were initially green and hard in texture (Figure 2C). The calli tissues appeared to be more prone to give rise to somatic embryos when the calli tissues became white and flaky in texture (Figure 2D). It was difficult to assess a change in fatty acid

96

composition at this stage because the calli cells did not differentiate and/or proliferate uniformly (Figures 2B-2C). The next transgenic tissues examined were somatic embryos, which were induced to develop from transgenic calli (Figure 2D). The somatic embryos were white at the earlier developmental stages and turned green upon maturation and exposure to light (Figures 2D-2F). We were able to detect a change in the fatty acid profiles of somatic embryos overexpressing the *FatB* cDNA (Figure 3). Unlike the calli cells, somatic embryos represent the first transgenic tissues available for analysis in which the cells are organized (for cell differentiation from calli tissues to occur) and development seems to be uniform once the somatic embryos began to elongate (Figures 2E-2F). The elongating somatic embryos matured into plantlets, which were placed into magenta boxes as shown in Figure 2G. The plantlets were transferred to soil when they were several centimeters in length (Figure 2H). These plantlets do not develop protective cuticle layers when they are in the humid environment of the magenta boxes. Thus the plantlets that were transferred to soil are placed in plastic bags, which are gradually opened as the plantlets adjust to the less humid environment. Once the plantlets have hardened off (development of cuticle layer and root system), the plantlets are grown to mature cotton plants in the greenhouse. Pooled T_1 seeds and cotyledons of these germinated seedlings (Table I) and leaves of primary transformants (T_0, Table II) were also analyzed for changes in fatty acid compositions. Transformations of the cotton explants with the anti-sense expression construct (ASPATE) gave rise to 25 primary transformants. About 30% (8 plants) of these ASPATE plants have a dwarf phenotype (Figure 7). To date, the transformations of cotton explants with the sense expression

97

construct (PATE) have given rise to only 5 primary transformants. These transformants are characterized in the following sections.

Overexpression of FatB in Transgenic Somatic Embryos

To manipulate the expression of FatB, transgenic cotton plants were produced harboring the sense PATE expression construct under the transcriptional control of the CaMV 35S promoter (Figure 1A). Somatic embryos, which developed from calli (Figure 2) following transformations with *Agrobacterium* harboring this construct were analyzed for changes in fatty acid composition. Somatic embryos overexpressing the *FatB* cDNA had a fatty acid composition that was different from vector-only (pBI 121) control. Two transgenic lines, PATE 717d and PATE jj-1 had up to 70% (of the total) palmitic acid (16:0, red bars) while vector-only controls had only about 25% palmitic acid (Figure 3), similar to wild-type levels. Elevated levels of stearic acid (18:0, green bars, up to 20% of the total) also were observed in these two transgenic lines. As expected there was a corresponding reduction in oleic acid (18:1, yellow bars) and linoleic acid (18:2, blue bars, Figure 3) levels. In addition, developing zygotic embryos (arising from normal fertilization *in planta*) from about 25 to 55 days post anthesis (dpa), have about 25% palmitic acid throughout seed maturation and reserve accumulation (Figures 3 and 4). Even during the period of maximum oil reserve accumulation (30-35 dpa), the relative percentage of palmitic acid remains unchanged (Figure 4). This may suggests that the endogenous levels of palmitic acid are tightly regulated such that a constant proportion of palmitic acid is essential at all times during development for the plant's growth and survival.

Stages of Plant Transformation and Tissue Culture

A. Cotton Cotyledon

B. Induction of Calli Tissue
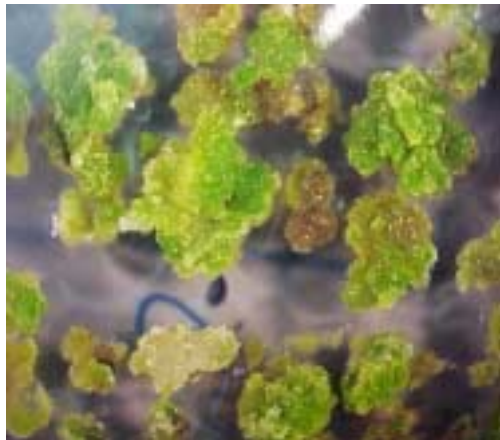
C. Calli Maturation

D. Induction of Somatic

Stages of Plant Regeneration

E. Embryo Maturation - Stage

F. Embryo Maturation - Stage

G. Plantlet Maturation

H. Plant Regeneration

Figure 2: Stages of transformation, tissue culturing, and plant regeneration. (A) Cotton explants on G1 medium for 3d after co-cultivation with *Agrobacterium tumefaciens* LBA4404, harboring the binary vector pBI 121 for vector-only control experiments or PATE sense or anti-sense constructs for fatty acid modification (transformations initiated by Shea Brown). (B) Explants transferred to G2 medium for the induction of calli formation. (C) The transformed calli are transferred to fresh G2 medium every 2-4 weeks. (D) After 2-6 months, proliferating transgenic calli were transferred to MSOB medium for the induction of somatic embryos. (E) Developing embryos are recovered after 6-8 weeks and placed on MSOB medium without antibiotics. (F) Elongated embryos were transferred to MS3 medium for root formation. (G) Plantlets (about 1-2 cm) were transferred to magenta boxes for continued growth on MS3 medium. (H) Plantlets (about 4-5 cm) were transferred to soil and hardened off prior to growth under greenhouse conditions.
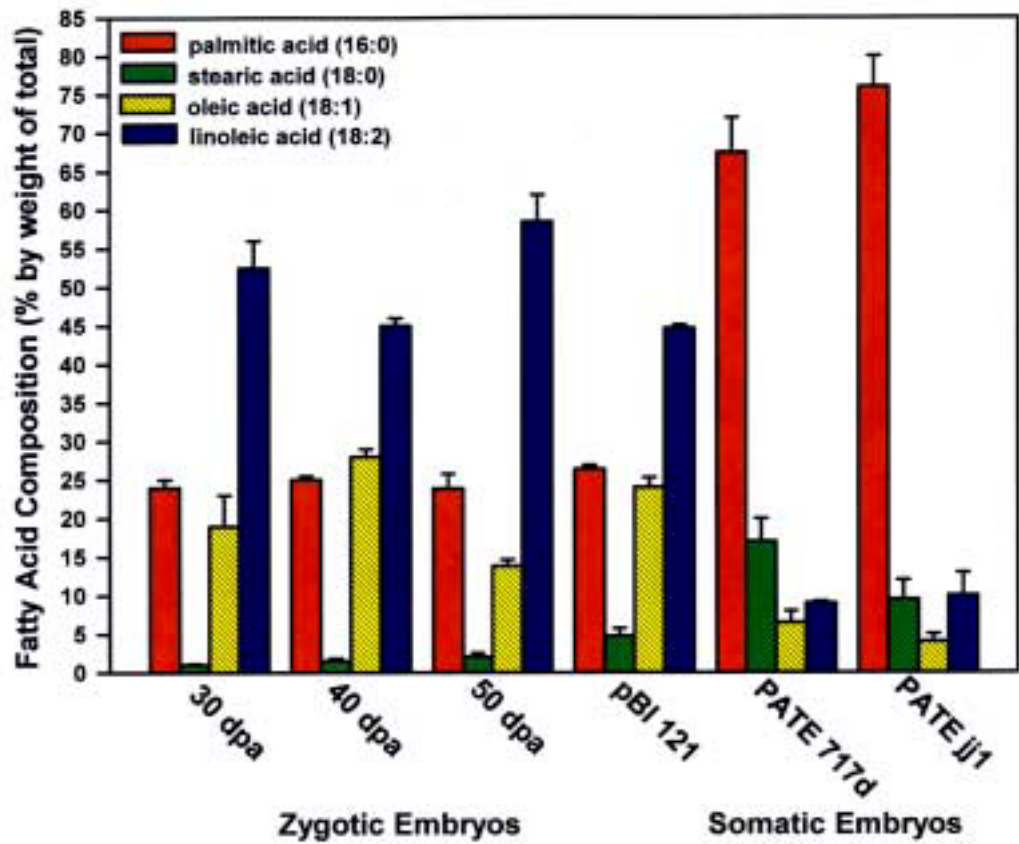
Figure 3: Comparison of the fatty acid composition of total lipids extracted from wild-type developing zygotic embryos (30-50 dpa) and transgenic somatic embryos. PATE 717d and PATE jj-1 somatic embryos developed from calli following transformations with *Agrobacterium* harboring constructs, which has the *FatB* cDNA sequence in sense

orientation downstream from the CaMV 35S promoter. pBI 121 somatic embryos developed from calli following transformations with *Agrobacterium* harboring the binary vector pBI 121 for vector-only control experiments. Total lipids extracted from these embryos were transesterified and FAMEs of palmitic acid (red), stearic acid (green), oleic acid (yellow), and linoleic acid (blue) were analyzed by GC-FID. Values represent the mean and standard deviation from three or more independent samples.

Detection of the FatB polypeptide

Polyclonal anti-peptide (RPKHAKSSANMDQITAKR, amino acid #395-412)

antibodies described in Chapter 2 were used to detect FatB proteins in cell-free extracts

of somatic embryos and zygotic embryos (Figure 5). Immunoblots probed with anti-FatB

antibodies recognized higher amounts of the 37 kDa FatB protein in somatic embryos of

transgenic lines, PATE 717d (S1) and PATE jj-1 (S2) compared to vector-only controls

(pBI 121, V) and 30 dpa zygotic embryos (Z). This apparent molecular weight was

estimated from plots of M_r in comparison with known protein molecular weight standards

(Hen egg white ovalbumin, 45 kDa; Bovine carbonic anhydrase, 31 kDa; Soybean trypsin

inhibitor, 21 kDa; Figure 5). The apparent molecular weight also is consistent with the

predicted molecular weight of the mature protein of 36.7 kDa (using Hitachi DNAsis

software). The FatB polypeptide also was recognized in crude homogenates of the

transgenic calli from the overexpressed sense PATE lines, PATE 717d (C1) and PATE jj-

1 (C2). No immuno-reactive proteins were detected on blots probed with the preimmune

serum. The smaller molecular weight proteins seen in the PATE 717d somatic embryo

(S1) and PATE 717d calli (C1) lane probably represents a proteolytic degradation

product, since its relative abundance differed from preparation to preparation or with

extended freeze-thaw cycles. Higher levels of the FatB protein detected immunologically

in somatic embryos support the notion that indeed FatB is overexpressed in these

transgenic PATE lines and that the overexpression of this enzyme resulted in the
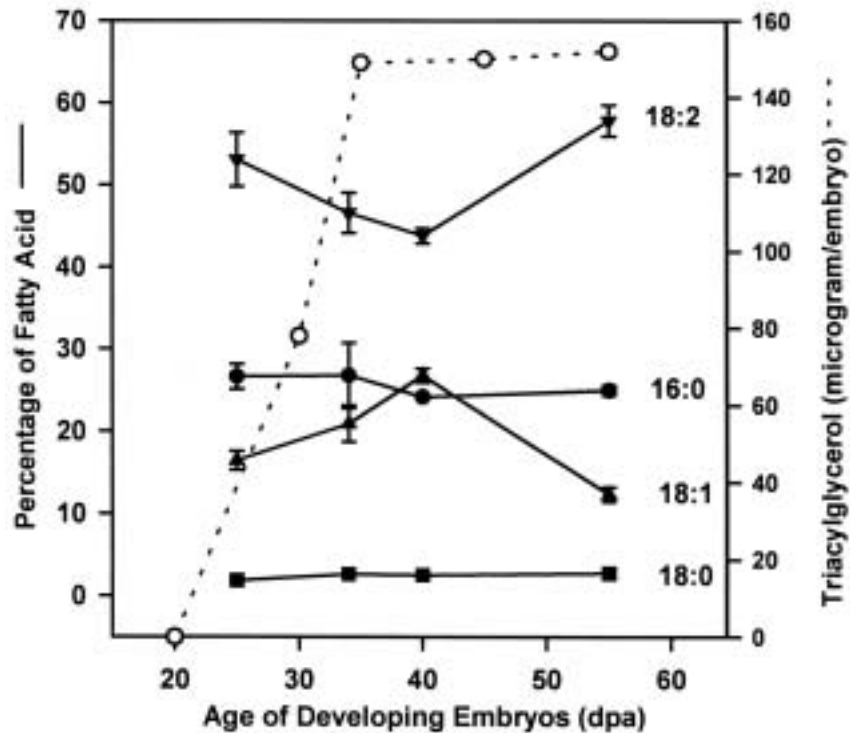
Figure 4: Fatty acid composition of developing zygotic embryos (25-55 dpa). Total lipids extracted from developing embryos were transesterified and FAMEs of palmitic acid (circle), stearic acid (square), oleic acid (triangle), and linoleic acid (inverted triangle) were analyzed by GC-FID. Triacylglycerol (dashed line) data are from Figure 5 of Chapter 1). Values represent the mean and standard deviation from three or more independent samples.
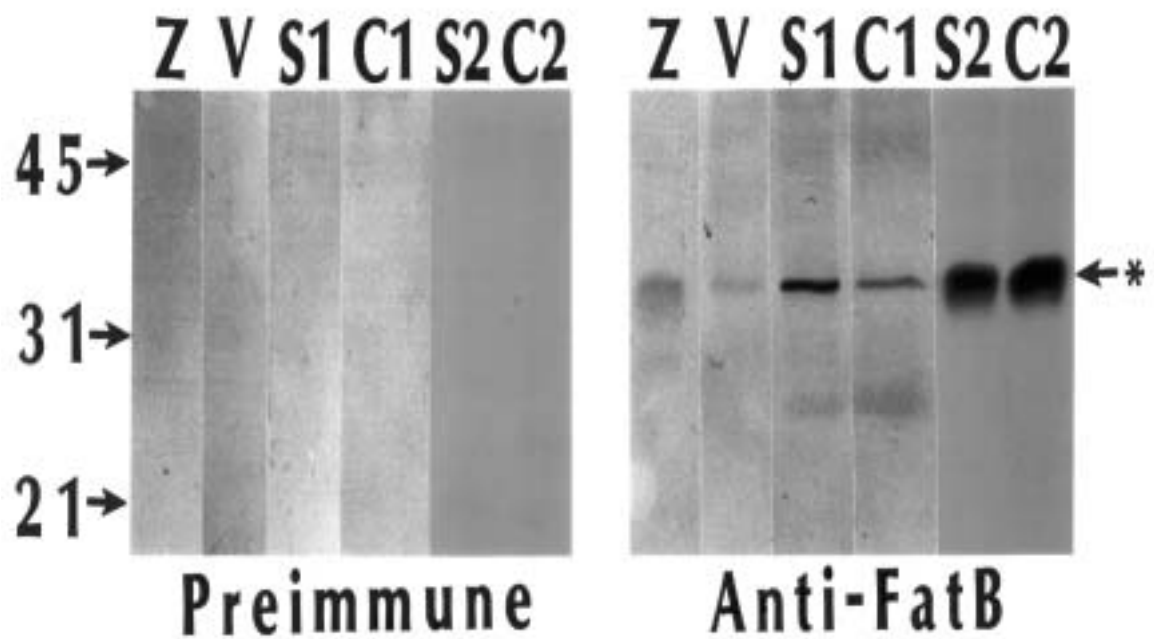
Figure 5: Immunological detection of the endogenous cotton FatB in 30 dpa zygotic

embryos (Z) and expression of the cotton FatB in transformed somatic embryos of vector

only control (pBI 121, V) and FatB overexpressed lines such as PATE 717d (S1) and

PATE jj-1 (S2), and in transformed calli of these lines (PATE 717d (C1) and PATE jj-1

(C2)). Immunoblots were probed with 1:5,000 preimmune serum or 1:5,000 anti-FatB

antibodies. The asterisk represents the 37 kDa antigen in cotton homogenates of

transformed and nontransformed cell-free extracts. The migrations of 21 kDa, 31 kDa,

and 45 kDa standard proteins are indicated by arrows on the left.

elevated levels of palmitic acid in these tissues (Figure 3). Thus based on the fatty acid profiles and the immunoblot analyses, we concluded that the overexpression of FatB directly influenced the change in fatty acid composition of the transgenic somatic embryos examined.

Phenotype of plants overexpressing FatB

Transgenic PATE plantlets were grown on sucrose medium (Figure 2G and Figure 6D) until they were about three to four centimeters tall. Then they were transferred to soil to harden off (development of protective cuticle and development of a more extensive root system) prior to growth in greenhouse conditions. After about two weeks off the sucrose-rich medium, the leaves of the PATE transgenic plants (Figures 6A-C) lost chlorophyll and senesced. The low amounts of polyunsaturated fatty acids (Figure 3) observed in these transgenic plants indicate that less polyunsaturated fatty acids are available for incorporation into membranes lipids and thus may affect the integrity of the chloroplast membranes. Consequently, the chloroplast membranes may be leaky and directly reduce photosynthetic efficiency of these leaves.

Alternatively, high amounts of free fatty acid accumulating in the cells of leaves may also be a contributing factor for premature chlorosis and leaf senescence. A significant increase in palmitic acid was detected in the somatic embryos of these PATE plantlets (Figure 3). Excess amounts of the palmitic acid in the cells could act as detergent to solubilize the chloroplast membranes. Ultimately, the complement of the

PATE Plantlets in Soil vs. on Sucrose Media

A. PATE jj-1 in soil

B. PATE 717d-1 in soil

C. PATE 717d-2 in soil

D. PATE 717d on sucrose media
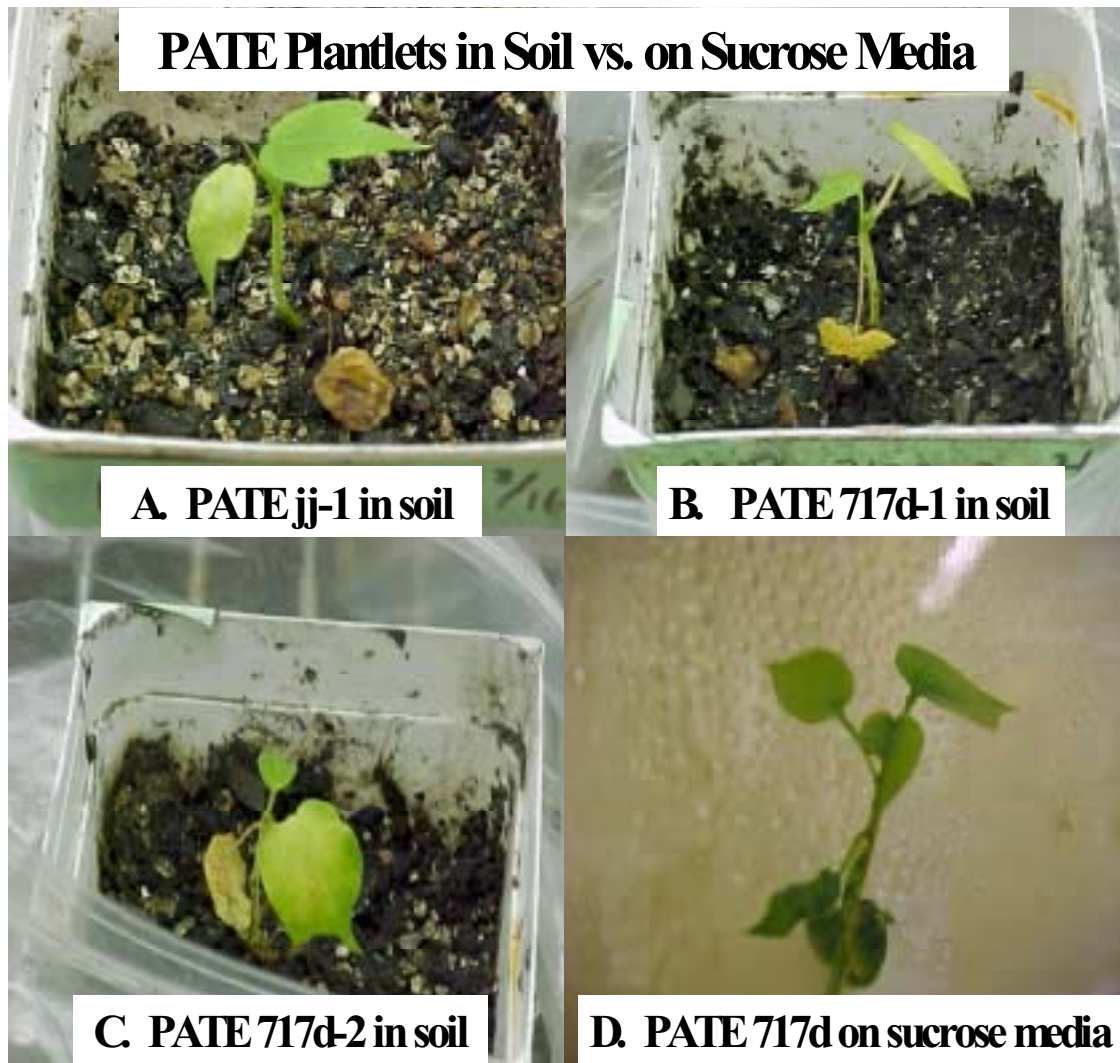
Figure 6: Leaves of transgenic PATE plantlets begin to senesce (A-C) several weeks after removal from sucrose-rich medium (D).

Table 1: Fatty acid composition of ASPATE pooled T_1 seeds and cotyledons of 2d-old cotton seedlings. Total lipids extracted from these tissues were transesterified and analyzed by GC-FID. Values represent the mean and range of at least two independent samples.

Samples	16:0	18:0	18:1	18:2
Pooled T_1 Seeds				
Coker 312	27.9±0.5	2.5±0.5	19.3±0.4	51.3±0.6
pBI 121	27.8±0.2	2.9±0.2	17.5±0.8	51.8±0.8
ASPATE 9B	26.4±0.6	2.8±0.2	14.8±1.8	54.3±1.9
ASPATE 16B	25.2±0.1	2.6±0.2	14.6±1.6	57.3±1.9
ASPATE 4C-3	28.9±0.9	2.9±0.3	15.1±0.8	49.6±1.3
Cotyledons				
Coker 312	27.8±0.2	2.9±0.4	17.3±1.9	52.7±1.4
pBI 121	27.3±0.6	2.7±0.2	16.4±1.1	53.5±1.7
ASPATE 9B	26.4±0.9	3.2±0.1	15.4±0.4	55.1±1.3
ASPATE 16B	27.8±1.2	2.2±0.3	13.2±2.4	56.9±3.9
ASPATE 4C-3	29.9±0.5	2.7±0.1	14.9±1.6	52.6±1.9

Table 2: Fatty acid composition of ASPATE Normal and Dwarf Phenotype leaves (of mature plants, T_0 primary transformants) compared to leaves of control plants. Total lipids extracted from leaves were transesterified and analyzed by GC-FID. Values represent the mean and range of at least two independent samples.

Samples	16:0	18:0	18:1	18:2	18:3
Controls:					
Coker 312	26.1±0.3	2.9±0.1	4.9±0.1	21.6±0.2	38.8±1.0
pBI 121	28.7±0.2	2.9±0.2	4.5±0.5	16.5±5.4	41.6±3.8
ASPATE Normal					
9B	25.0±1.0	2.9±0.1	5.9±1.2	22.9±1.7	36.8±2.6
16B	23.2±0.9	3.1±0.3	4.2±0.7	17.3±1.0	46.9±1.9
4	25.1±0.2	3.7±0.2	4.9±0.1	14.6±0.8	47.1±4.3
ASPATE Dwarf					
1A-1	31.2±3.3	3.8±0.5	4.6±0.3	20.8±2.9	46.5±1.9
7	31.5±4.5	4.8±1.2	6.5±1.4	13.8±5.9	44.5±0.7
15A-1	25.2±1.8	4.5±1.4	4.7±1.2	15.4±4.9	41.1±1.7

different lipid acyl chains most certainly is important for the functional integrity of membranes, and as such the normal growth and development of these plants appear to be severely compromised.

Expression of the anti-sense *FatB* cDNA

Transgenic cotton plants also were produced harboring the anti-sense PATE (ASPATE) expression construct under the transcriptional control of the CaMV 35S promoter (Figure 1B). Transgenic lines such as ASPATE 9B, ASPATE 16B, and ASPATE 4C-3 appeared phenotypically similar to the wild-type plants (Coker 312 cv.) and vector-only (pBI 121) control transgenic plants. The fatty acid composition was examined in samples of pooled T_1 seeds and cotyledons of 2-d-old cotton seedlings (T_1 individuals; Table 1). No substantial change in the relative percentage of palmitic acid was observed in any of the tissues examined. Palmitic acid levels ranged from 23 to 30% of the total in seeds and cotyledons. In addition, expression of the anti-sensed *FatB* cDNA in leaves of mature plants (T_0 primary transformants, Table II) did not result in a reduction of palmitic acid percentages when compared to control plants. Based on these data, we suspected that there was some regulatory mechanism in cotton such that it was not possible to reduce the relative percentage of palmitic acid in tissues of transgenic plants. This concept, although difficult to test, suggested that cotton plants maintained a constant percentage of palmitic acid for membrane lipid biosynthesis, and that a reduction in palmitic acid content was manifested in smaller plant cell size.

Dwarfed plants harboring the anti-sense *FatB* construct

Interestingly, about 30% of the anti-sensed transgenic plants displayed a "dwarf"

phenotype, while the others had a more "normal" phenotype similar to wild-type. The

over-all size of these dwarf plants was significantly reduced. The normal phenotype

plants grew as tall as five feet while the dwarf plants were shrub-like and were only about

one to two feet tall (Figure 7A). The number and size of the bolls on these dwarf plants

also were reduced. The bolls on dwarf plants, such as ASPATE 2E, were smaller in size

compared to bolls of wild-type, unaffected ASPATE, or vector-only (pBI 121) control

plants (Figure 7B). In addition the bolls of dwarf plants only had one to two locules with

only one seed per locule, whereas typically bolls would have about four to five locules

with about five seeds per locule (Figure 7C).

Another quantifiable difference was observed in the size of the mature leaves

(Figure 8A-B). The mid-vein lengths were measured and used to determine if the

reduction in leaf sizes of the dwarf plants were statistically different than the normal

phenotype plants and controls. Dwarf transgenic lines, ASPATE 1A-1, ASPATE 2E,

ASPATE 7, and ASPATE 15A-1, had significantly smaller mature leaves, which had

mid-veins that were only three to four centimeters in length compared to those of normal

phenotype and control plants ($P<0.0001$). Normal phenotype transgenic lines, ASPATE

9B, ASPATE 16B, and ASPATE 4, and control plants (wild-type Coker 312 cv. and

vector-only pBI 121) had mid-veins in the range of about six to nine centimeters in

length. Younger leaves (about 10 days after emergence, Figure 8C) of dwarf plants also

were significantly smaller ($P< 0.0001$). At this developmental stage, the mid-veins of

A. ASPATE 9B vs. ASPATE 2E

B. ASPATE2E, ASPATE9B, pBI121

C. ASPATE2E, ASPATE9B

Figure 7: ASPATE plants displaying normal and dwarf phenotypes. (A) Dwarf ASPATE 2E plant is shrub-like and significantly shorter than the normal phenotype ASPATE 9B plant. (B) Dwarf plants (ASPATE 2E) have smaller bolls compared to normal phenotype plants (ASPATE 9B) and vector-only control plants (pBI 121). (C) The dwarf bolls produce fewer locules with fewer viable seeds.
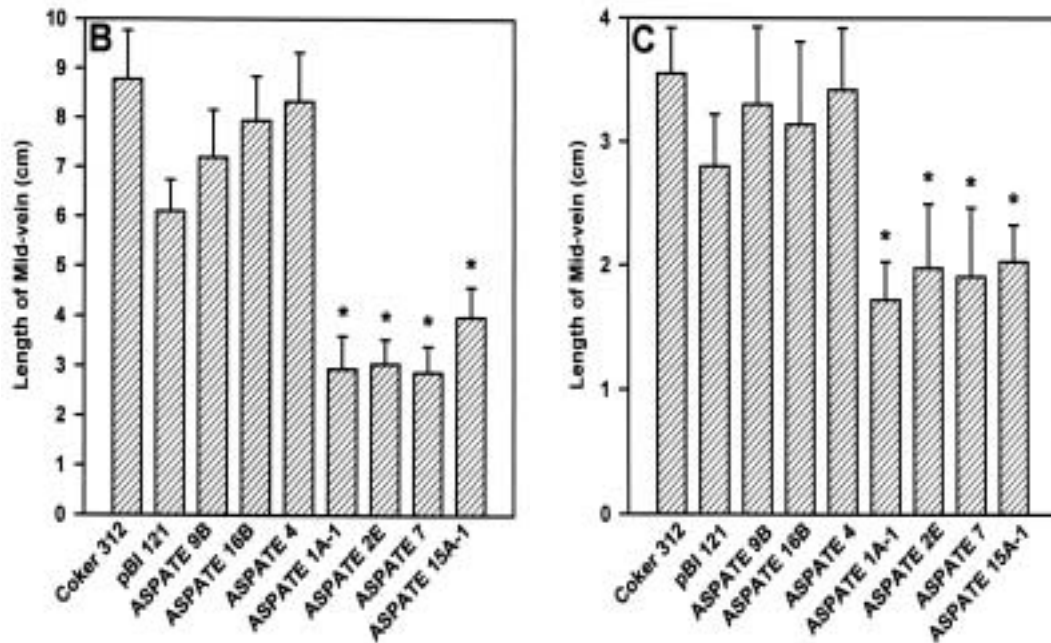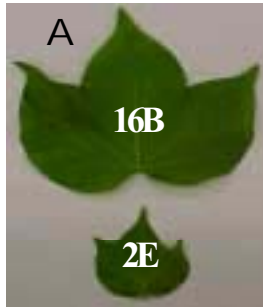
Figure 8: Mid-vein lengths of young and mature leaves from dwarf phenotype ASPATE

plants compared to normal phenotype ASPATE plants and control plants (wild-type,

Coker 312 cv. and vector-only control, pBI 121). (A) The over-all sizes of the mature

leaves of ASPATE plants are indicative of which phenotype they possess. ASPATE

plants obviously display a normal phenotype (ASPATE 16B and ASPATE 9B) or dwarf

phenotype (ASPATE 2E and ASPATE 15A-1). (B) The mid-vein length (cm) of mature

leaves on ASPATE dwarf plants (ASPATE 1A-1, 2E, 7, 15A-1) are significantly shorter

than mature leaves of normal phenotype ASPATE plants (ASPATE 9B, 16B, 4) and

control plants (wild-type, Coker 312 cv and vector-only control, pBI 121). Asterisks

indicate a p-value<0.0001. Values represent the mean and standard deviation of at least

20 leaves per plant. (C) The mid-vein length (cm) of young leaves (about 10 days about

emergence) of the same plants described in panel B. Asterisks indicate a p-value<0.0001.

Values represent the mean and standard deviation of at least 20 leaves per plant.
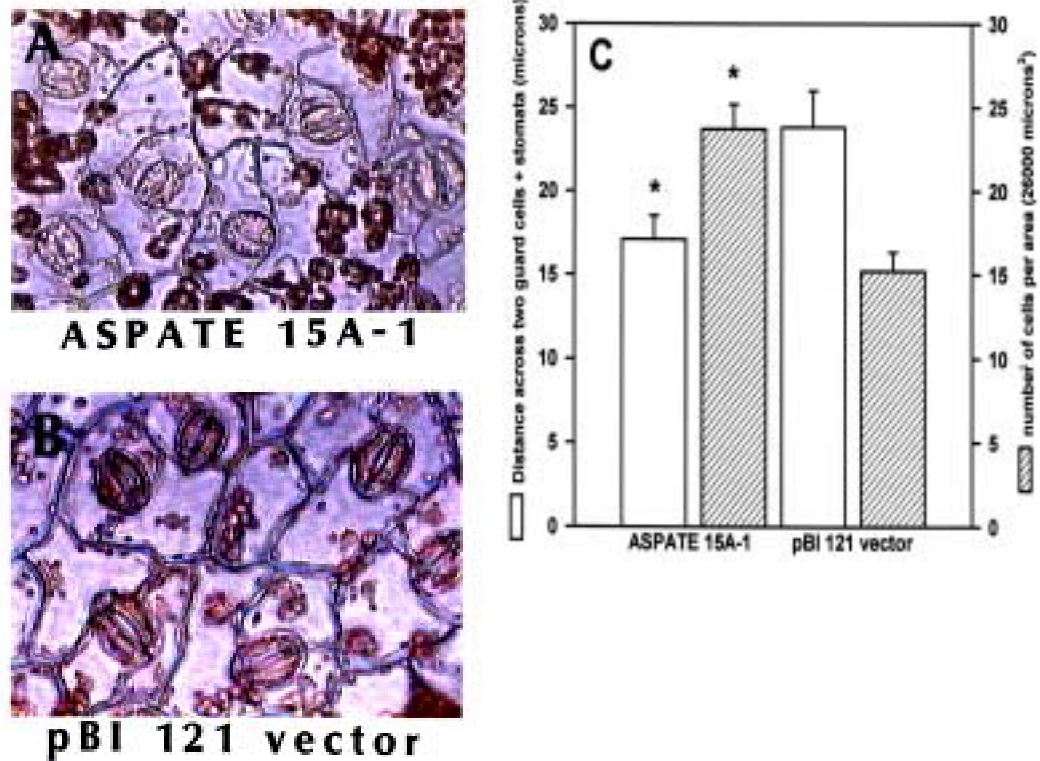
Figure 9: Dwarf ASPATE plants (e.g. ASPATE 15A-1) have smaller leaf epidermal cells including smaller guard cells and stomata compared to vector-only control (pBI 121) plants. (A, B) Images of eosin-stained leaf epidermal cells at 250X magnification. (Area of view is representative of 26000 microns^2). (C) The distance (open bars) across two guard cells including the stomata was measured. Values represent the mean and standard deviation of at least 20 pairs of guard cells from 5 leaves of each plant. Asterisks indicate a p-value<0.001. The number of epidermal cells (hatched bars) also were quantified per unit area (26000 microns^2). Values represent the mean and standard deviation of about 50-100 cells counted from three different leaves. The asterisk indicates a p-value<0.001.

dwarf leaves were only about one centimeter shorter than the other plants. Perhaps there was a reduced supply of palmitic acid in the dwarf plants for cell expansion in leaves, and as such, there is a more dramatic difference in leaf size at maturity (fully expanded). In addition, no change in palmitic acid percentage was detected in the dwarf leaves when compared to leaves of ASPATE Normal phenotype and control plants (Table 2). Thus, the overall percentage of palmitic acid remains unchanged, but a reduced amount of palmitic acids levels may have contributed to the smaller cell size observed in dwarf plants. The leaves of dwarf plants also had smaller epidermal cells including smaller stomata and guard cells (Figure 9A) compared to leaves of the vector-only control plants (pBI 121, Figure 9B). Comparing the distance across two guard cells, including the stomata opening, revealed that the leaves of dwarf plants had significantly smaller cells (Figure 9C). The distance across two guard cells in a dwarf transgenic line such as ASPATE 15A-1 was about 17 microns while the distance measured for the vector-only control cells was about 26 microns (P< 0.001). In addition, there was a higher number of epidermal cells per unit area (26000 microns^2) in the leaves of dwarf plants (about 25 cells) compared to leaves of control plants (only about 17 cells), supporting that the cells of dwarf plants generally were significantly smaller (P<0.001) than those of normal control plants. Similar values were quantified in other ASPATE dwarf lines.

Transgenes were incorporated into the cotton genome

To confirm that the transgenes were incorporated into the plant genome of transgenic cotton plants, a PCR strategy (Figure 10A) was devised based on the advantage that both genomic and cDNA sequences of the *FatB* gene were published

(Pirtle et al., 1999; Yoder et al., 1999). PCR amplimers were designed to amplify a DNA product of 715 bp encompassing the DNA sequence between Exon 3 and Exon 5 (including Exon 4 and Introns 3 and 4) of the endogenous *FatB* gene. Accordingly these PCR amplimers would amplify a PCR product of about 354 bp in the corresponding cDNA sequence between Exons 3 and 5 (including Exon 4) of the cDNA-derived transgene if it was incorporated into the plant genome. Consequently, transgenic plants would have both PCR products, one representing the amplified portion of the endogenous gene and the other product representing the amplified portion of the incorporated transgene.

DNA samples were isolated from young leaves of wild-type plants (Coker 312 cv.), vector-only control plants (pBI 121), and various transgenic lines of normal phenotype plants such as ASPATE 9B, ASPATE 16B, and ASPATE 4C-3 and dwarf phenotype plants such as ASPATE 2E, ASPATE 7, and ASPATE 15A-1 (Figure 10B). Plasmid DNA (PATE cDNA ligated into pUC19 vector) served as a control and representative of the 354 bp PCR product (Lane 1). As expected, only the 715 bp PCR product was amplified from DNA of wild-type Coker 312 plants (Lane 2) or vector-only control plants (Lane 3). All transgenic lines analyzed had both the 354 bp and 715 bp PCR products indicating that these lines had the transgene incorporated into their genome (Lanes 4,6,8-11). Progeny lines of ASPATE 9B (Lane 5) and ASPATE 16B (Lane 7) also were analyzed. The DNA isolated from the leaves of these T_2 plants were used in similar PCR reactions resulting in the presence of both PCR products as well. This indicates that the transgene was stable through the second generation (after the first
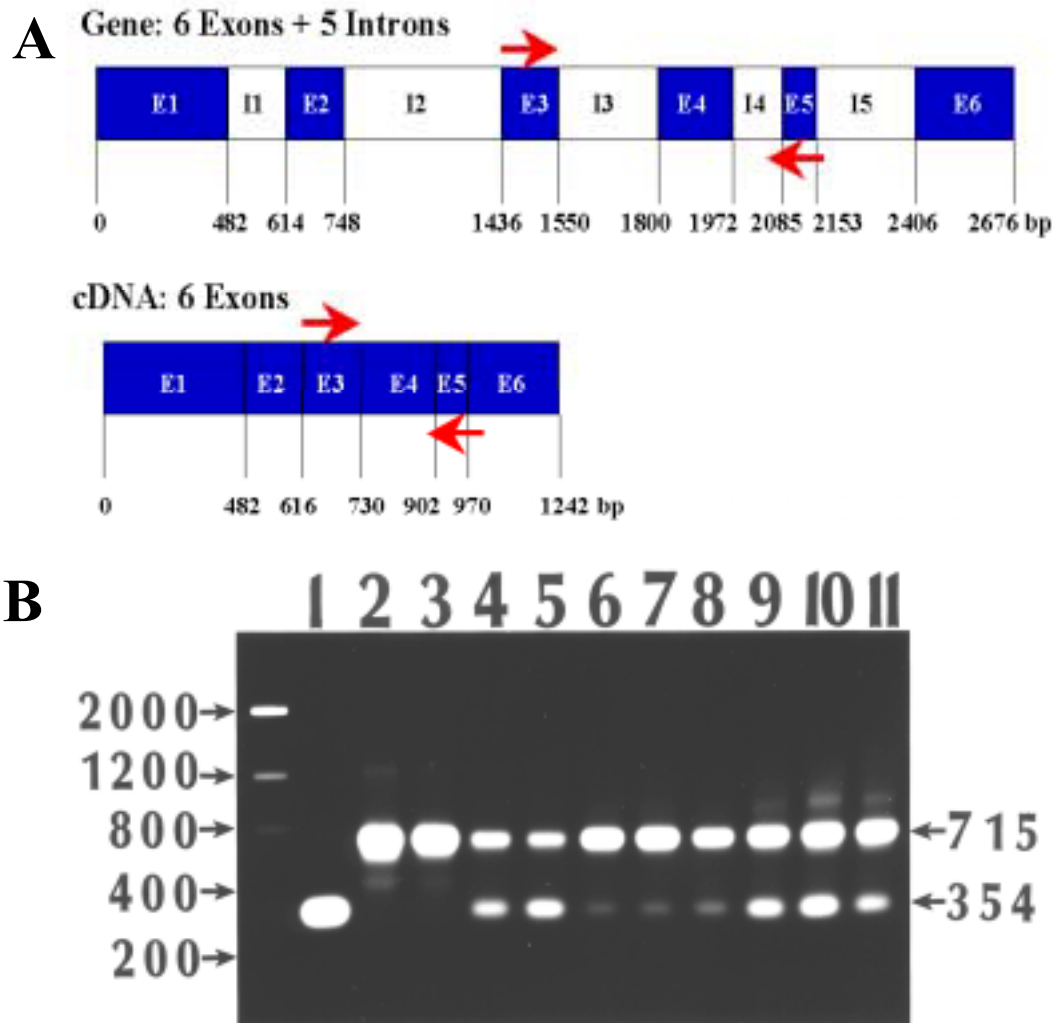
Figure 10: Confirming the incorporation of the transgene in the plant genome. DNA (300 ng) isolated from young leaves of various primary (T_1) anti-sensed plants and control plants (wild-type, Coker 312 cv. (Lane 2) and vector-only control, pBI 121 (Lane 3) was used in PCR reactions. DNA from normal phenotype plants analyzed include ASPATE 9B (Lane 4), ASPATE 16B (Lane 6), ASPATE 4C-3 (Lane 8) and dwarf

phenotype plants include ASPATE 2E (Lane 9), ASPATE 7 (Lane 10), ASPATE 15A-1 (Lane 11). The DNA from progeny (T$_2$) plants (ASPATE 9B, Lane 5 and ASPATE 16B, Lane 7) also was analyzed. (A) The PCR strategy involved the design of PATE forward and reverse PCR primers to amplify a 715 bp PCR product from DNA samples with only endogenous genes and the 715 bp PCR product plus a 354 bp PCR product from DNA samples with both endogenous genes and the transgene. (B) The expected PCR products were visualized with ethidium bromide staining following electrophoresis in 1.5% agarose gels. Plasmid DNA (PATE cDNA ligated into pUC19 vector) was loaded in lane 1 as a control and representative of the 354 bp PCR product. The migrations of 200 bp to 2000 bp DNA standard fragments is indicated by arrows on the left. The arrows on the right indicate the migrations of the 715 bp and 354 bp PCR products.

segregation event). Collectively data from these PCR experiments revealed that both phenotypes of ASPATE plants had the incorporation of the transgene in their genome thus ruling out the possibility that the normal phenotype plants did not have the transgene. Based on these data we are still unclear about the mechanism, which dwarfed some of the ASPATE plants. Perhaps, it may be detrimental to reduce the proportion of palmitic acid below a certain range such that the surviving normal phenotype ASPATE plants characterized here only have a slight reduction in overall palmitic acid. In addition, the dwarf ASPATE plants may have a more severe reduction in palmitic acid levels thus resulting in smaller plant features to compensate for the limitation in palmitic acid available for biomembrane synthesis and cell expansion.

Discussion

Previously, a cotton (*Gossypium hirsutum* L.) *FatB* cDNA expressed in *E. coli* was shown to encode an acyl-ACP thioesterase with activity toward palmitoyl-ACPs (Chapter 2). *In vitro* assays and the quantification of free fatty acids accumulating within *E. coli* revealed that the expressed FatB enzyme preferably produced 16:0 free fatty acids from acyl-ACPs.   In addition, *in vitro* acyl-ACP thioesterase activity of crude cotton homogenates indicated that activity toward 16:0-ACPs predominated over activity toward $18:1^{\Delta9}$-ACPs (Chapter 1; Pirtle et al., 1999). Furthermore, the palmitoyl-ACP thioesterase activity was highest in developing embryo extracts at the maximum rate of reserve accumulation (oil and protein; 30-35 dpa) indicating that this enzyme may be responsible for the high level of palmitic acid incorporated in cottonseed oil (Chapter 1).

Thus we speculated that this FatB thioesterase plays a primary role in the incorporation of extraplastidial, saturated fatty acids in some cotton tissues. However, the *in planta* role of FatB thioesterases are still unclear. To examine the *in planta* activity of the palmitoyl-ACP thioesterase (FatB) in cotton, we produced transgenic cotton plants harboring sense and antisense FatB expression constructs (Figure 1) under the transcriptional control of the CaMV 35S strong, constitutively active promoter. We were able to manipulate the expression of the FatB thioesterase and alter palmitic acid levels in some cotton tissues (Figure 3).

Immunoblots probed with anti-FatB antibodies recognized substantially higher amounts of the 37 kDa FatB protein in cell-free extracts of transgenic somatic embryos (harboring the PATE sense overexpression construct) compared to vector-only (pBI 121) control embryos (Figure 5) or zygotic embryos of wild-type plants. Overexpression of FatB in these transgenic somatic embryos resulted in elevated levels of palmitic acid (up to 70% of the total; Figure 3) indicating that the increase in FatB proteins directly influenced the amount of palmitic acid distributed to these tissues. There was almost three times more palmitic acid in these transgenic somatic embryos than is normally found in zygotic embryos. Developing zygotic embryos from about 25 to 55 dpa maintain a relatively constant palmitic acid content (about 25% of the total) throughout development (Figures 3 and 4), suggesting that palmitic acid distributed to these embryos may be tightly regulated. Perhaps the right proportion of palmitic acid in these tissues is essential for normal development and growth.

The notion that the correct proportion of palmitic acid is important for plant development was evident when the plantlets overexpressing FatB were removed from sucrose-rich medium and placed into soil (Figures 2 and 6). These plantlets developed from somatic embryos, which have high levels of palmitic acid and very low levels of unsaturated fatty acids (Figure 3). The somatic embryos have less than 20% of the unsaturated fatty acids (18:1 and 18:2), which are major components of lipids in the thylakoid membranes. Low levels of unsaturated fatty acids can contribute to the leakiness of the chloroplast membranes such that photosynthetic efficiency is greatly reduced (Somerville et al., 2000). The presumed nonfunctional chloroplast membranes also may be due to the accumulation of excess free palmitic acid in the chloroplasts of plant cells, which can act as a detergent to solubilize membranes. The leaves of the PATE plantlets quickly lost their chlorophyll and senesced after the removal from the sucrose medium (Figure 6) suggesting that the plantlets could not survive autotrophically due to the less efficient photosynthetic machinery. The same phenomenon was observed in *Arabidopsis fad*2 *fad*6 mutants, which lacked diunsaturated fatty acids (Somerville et al., 2000). These mutants lost nearly all photosynthetic capacity and also could not grow autotrophically. However, when these mutant plants were placed on sucrose media, photosynthesis was restored and organ development seemed to be normal. This indicates that there is a direct correlation between the fatty acid composition in cells and the normal development of the plant. Collectively, our data revealed that overexpressing FatB in transgenic cotton plants directly increased the supply of palmitic acid distributed within cells of somatic embryos. As a consequence, lower levels of unsaturated fatty

acids or higher levels of free fatty acids reduced the photosynthetic efficiency and thus hindered the development of these PATE plants.

To further examine the *in planta* role of the FatB, we characterized the transgenic plants harboring the anti-sense PATE (ASPATE) expression construct under the transcriptional control of the CaMV 35S promoter (Figure 1). These ASPATE plants displayed two obvious phenotypes: a "normal" phenotype similar to wild-type plants (Coker 312 cv.) and vector-only control plants (pBI 121) and a smaller "dwarf" phenotype. The normal phenotype plants such as ASPATE 9B, ASPATE 16B, and ASPATE 4C-3, had total fatty acid compositions in pooled T_1 seeds, cotyledons of 2 d-old seedlings (of T_1 individuals Table 1), and leaves of mature plants (T_0 primary transformants, Table 2) which were similar to wild-type and vector-only control fatty acid profiles. In addition, expression of the anti-sense *FatB* cDNA in somatic embryos did not show a reduction in the percentage of palmitic acid compared to vector-only control somatic embryos (data not shown). We originally suspected that these normal phenotype lines may not be transgenic, but PCR amplification (Figure 10) of DNA isolated from these plants revealed that the transgene (represented by the 354 bp DNA product) was incorporated in the plant genome. Thus palmitic acid levels may be regulated such that it is not possible to reduce the palmitic acid level below a certain range without causing lethal effects, at least in the tissues examined.

About 30% of the ASPATE plants had a dwarf phenotype. PCR amplification of the DNA isolated from these plants also revealed that the transgene has been incorporated into the genome (Figure 10). These dwarf plants had smaller bolls with a much reduced

124

number of viable seeds (Figure 7) and smaller leaves (Figure 8) than the normal phenotype plants and control plants (wild-type, Coker 312 cv. and vector-only control, pBI 121). Dwarf transgenic lines such as ASPATE 1A-1, ASPATE 2E, ASPATE 7, and ASPATE 15A-1, all had smaller leaves (of mature T_0 primary transformants) with smaller epidermal cells, including smaller guard cells and stomata (Figure 8). We could not detect a change in the overall fatty acid composition in the T_1 seeds of dwarf plants (data not shown) or leaves (of mature T_0 primary transformants, Table 2) when comparing equivalent amounts of dwarf ASPATE tissues to control tissues. Even though the dwarf plants only had one to two viable seeds per boll compared to normally twenty to twenty five seeds per boll, a single seed from the dwarf plants had the same fatty acid composition as observed in individual control seeds. This suggests that a reduction in palmitic acid may force the plant to distribute an adequate amount of palmitic acid to only one or two viable seed rather than aliquot a reduced amount of palmitic acid into many non-viable seeds. Furthermore, developing leaves of dwarf plants stopped leaf expansion earlier than the normal phenotype ASPATE plants (Figure 8), suggesting that a reduction in palmitic acid limited the amount of fatty acids available for incorporation into membrane lipids and thus hinder cell expansion. Alternatively the embryos that developed into apparently normal seeds could have been the result of a segregating "null" phenotype.

The PATE and ASPATE transgenic plants provided new insights into the influence of palmitic acid levels on normal plant growth and development. In the case with PATE transgenic plants, our data collectively revealed that overexpression of FatB

directly influenced the fatty acid composition of somatic embryos such that there were

elevated levels of saturated fatty acids (16:0 and 18:0) and reduced levels of unsaturated

fatty acids (18:1 and 18:2). The PATE plantlets once removed from the sucrose medium

could not grow autotrophically perhaps due to the low levels of unsaturated fatty acids or

high levels of free fatty acids, which both could directly affected the integrity of the

chloroplast membranes. It is possible that the thylakoid membranes lacked the proper

composition of membrane lipids to be photosynthetically efficient and thus plant

development was hindered. One future research aim involves the supply of sucrose to the

PATE plants in attempts to restore normal development as seen with the *Arabidopsis*

*fad*2 *fad*6 mutants. Or perhaps by supplying exogenous palmitic acid, these plants (or

leaves of these plants) could be restored to wild-type phenotype.

In the case with the ASPATE transgenic plants, we did not observe any changes

in the fatty acid composition of the tissues examined. However, the dwarf plants had

fewer viable seeds and smaller leaves possibly suggesting that a reduction in palmitic

acid affected embryo development and limited leaf expansion. One future research goal

with these dwarf plants involves spraying the plants with palmitic acid in attempts to

restore the "normal" phenotype (e.g. taller plants, more viable seeds, and larger mature

leaves). These developmental defects observed in ASPATE plants may be contributed to

the constitutive expression of the anti-sense FatB construct under the transcriptional

control of the CaMV 35S promoter. Although the CaMV 35S promoter gives rise to

strong expression in leaves (Dörmann et al., 2000), it has been reported that this promoter

is not highly active in the plant seeds (Eccleston and Ohlrogge, 1998). Though this was

reported to be the case in *Arabidopsis*, this also may be one potential explanation for the subtle changes in fatty acid composition of ASPATE pooled T_1 seeds (Table 1) and the smaller leaves (T_0 primary transformants) of dwarf ASPATE mature plants (Figure 8).

One potentially insightful experiment may be to produce transgenic cotton plants harboring the sense and anti-sense PATE expression construct under the transcriptional control of a seed-specific promoter. These plants should allow us to manipulate FatB expression specifically in seeds and to examine its influence on the fatty acid composition of seeds. Dörmann et al. (2000) reported the accumulation of higher proportions of palmitic acid in *Arabidopsis* seeds when the *ATFATB1* (*Arabidopsis thaliana* FATB1 thioesterase) cDNA was overexpressed under the transcriptional control of the seed-specific napin promoter. Futhermore, analyzing the physiological differences between transgenic plants generated from constitutive FatB expression compared to transgenic plants generated from seed-specific FatB expression will provide us with more comprehensive data revealing how palmitic acid levels influence normal plant growth and development. Nonetheless, the data presented in this chapter collectively revealed that the manipulation of FatB thioesterase expression altered levels of palmitic acid in some cotton tissues indicating that the FatB thioesterase directly plays a role in regulating palmitic acid levels.

Acknowledgments

References

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 12: 248-254.

Browse JA, McCourt PJ, Somerville CR (1986) A mutant of *Arabidopsis* deficient in C18:3 and C16:3 leaf lipids. *Plant Physiol* 81: 859-964.

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

Chapman KD, Trelease RN (1991) Acquisition of membrane lipids by differentiating glyoxysomes: role of lipid bodies. *J. Cell Biol.* 115: 995-1007.

Christie WW (1982) Lipid Analyses, 2nd ed. Pergamon Press, New York, pp. 52-54.

Dehesh K, Edwards P, Hayes T, Cranmer AM, Fillatti J (1996) Two novel thioesterases are key determinants of the bimodal distribution of the acyl chain length of *Cuphea palustris* seed oil. *Plant Physiol* 110: 203-210.

Dörmann P, Kridl J, Ohlrogge JB (1994) Cloning and expression in *Escherichia coli* of a cDNA coding for the oleoyl-acyl carrier protein thioesterase from coriander (*Coriandrum sativum* L.). *Biochim Biophys Acta* 1212: 134-136.

Dörmann P, Spener F, Ohlrogge JB (1993) Characterization of two acyl-acyl carrier protein thioesterases from developing *Cuphea* seeds specific for medium-chain and oleoyl-acyl carrier protein. *Planta* 189: 425-432.

Dörmann P, Voelker TA, Ohlrogge JB (1995) Cloning and expression in *E. coli* of a novel thioesterase from *Arabidopsis thaliana* specific for long chain acyl-acyl carrier proteins. *Arch Biochem Biophys* 36: 612-618.

Dörmann P, Voelker TA, Ohlrogge JB (2000) Accumulation of palmitate in *Arabidopsis* mediated by the acyl-acyl carrier protein thioesterase FATB1. *Plant Physiol* 123: 637-643.

Eccleston VS, Cranmer AM, VoelkerTA, Ohlrogge JB (1996) Medium-chain fatty acid biosynthesis and utilization in *Brassica napus* plants expressing lauroyl-acyl carrier protein thioesterase. *Planta* 198: 46-51.

Eccleston VS, Ohlrogge JB (1998) Expression of lauroyl-acyl carrier protein thioesterase in *Brassica napus* seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. *Plant Cell* 10: 613-622.

Fergusen DL, Turley RB, Meredith WR, Jr. (1996) Comparison of protein profiles during cotton (*Gossypium hirsutum* L.) fiber cell development with partial sequences of two proteins. *J. Agricultural and Food Chemistry* 44: 4022-4029.

Firoozabady E, DeBoer DL, Merlo DJ, Halk EL, Amerson LN, Rashka KE, Murray EE (1987) Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. *Plant Mol. Biol.* 10: 105-116.

Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochim Biophys Acta* 1301: 7-56.

Havaux M (1987) Effects of chilling on the redox state of the primary electron acceptor Q_A of photosytem II in the chilling-sensitive and resistant plant species. *Plant Physiol Biochem* 25: 735-743.

Hugly S, Somerville C (1992) A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. *Plant Physiol* 99: 197-202.

Kinney AJ (1994) Genetic engineering of the storage lipids of plants. *Current Opinion in Biotechnology* 5: 144-151.

Kinney AJ (1997) Genetic engineering of oilseeds for desired traits. *In* Genetic Engineering, vol 19, Setlow JK (ed.) Plenum Press, New York, pp. 149-166.

Kinney AJ, Knowlton S (1998) Designer oils: the high oleic soybean. *In* Genetic Modification in the Food Industry, Roller S, Harlander S (eds.) Blackie Academic and Professional, New York, pp. 193-213.

Miguel M, James D, Dooner H, Browse J (1993) *Arabidopsis* requires polyunsaturated lipids for low temperature survival. *Proc Natl Acad Sci USA* 90: 6208-6212.

Ohlrogge JB, Browse J (1995) Lipid biosynthesis. *Plant Cell* 7:957-970.

Ohlrogge JB, Jaworski JG (1997) Regulation of fatty acid synthesis. *Annu Rev Plant Physiol Plant Mol Biol* 48:109-136.

Ohlrogge JB, Jaworski JG, Post-Beittenmiller D (1993) De novo fatty acid biosynthesis. *In* Moore TS, Jr (ed.) Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 3-32.

Paterson AH, Brubaker CL, Wendel JF (1993) A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. *Plant Mol. Biol. Rep.* 11: 122-127.

Pirtle RM, Yoder DW, Huynh TT, Nampaisansuk M, Pirtle IL, Chapman KD (1999) Characterization of a palmitoyl-acyl carrier protein thioesterase (FatB1) in cotton. *Plant Cell Physiol* 40: 155-163.

Routaboul J, Fisher SF, Browse J (2000) Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol* 124: 1697-1705.

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

Somerville C, Browse J, Jaworski J, Ohlrogge JB (2000) Lipids. *In* Biochemistry and Molecular Biology of Plants, Buchanan B, Gruissem W, Jones R (eds.) American Society of Plant Physiologists, Rockville, MD, pp 456-527.

Thomas JC, Adams DG, Keppenne VD, Wasmann CC, Brown JK, Kanost MR, Bohnert HJ (1995) Protease inhibitors of Maduca sexta expressed in transgenic cotton. *Plant Cell Rep.* 14: 758-762.

Trelease RN, Doman DC (1984) Mobilization of oil and wax reserves. *In* Seed Physiology, vol 2, Murray DL (ed.) Academic Press, New York, pp 201-145.

Trolinder NL, Goodin JR (1988) Somatic embryogenesis in cotton (*Gossypium*): Requirements for embryo development and plant regeneration. *Plant Cell Tissue Org. Cult.* 12: 43-53.

Umbeck P, Johnson G, Baron K, Swain W (1987) Genetically transformed cotton (*Gossypium hirsutum* L.) plants. *Biotechnology* 5: 263-266.

Voelker T (1996) Plant acyl-ACP thioesterase: Chain-length determining enzymes in plant fatty acid biosynthesis. *In* Genetic Engineering, vol 18, Setlow JK (ed.) Plenum Press, New York, pp 111-133.

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

Wu J, Lightner J, Warwick N, Browse J (1997) Low temperature damage and subsequent recovery of *fab*1 mutant *Arabidopsis* exposed to 2°C. *Plant Physiol* 113: 347-356.

Yoder DW, Nampaisansuk M, Pirtle IL, Chapman KD, Pirtle RM (1999) Molecular cloning and nucleotide sequence of a gene encoding a cotton palmitoyl-acyl carrier protein thioesterase. *Biochim Biophys Acta* 1446: 403-413.

Summary and Significance

Acyl-chain elongation during fatty acid biosynthesis is terminated by acyl-ACP thioesterases when the thioester bond is cleaved. Subsequently the acyl groups are released from the ACPs (Harwood, 1988; Harwood, 1996) and exported to extraplastidic compartments. Previous characterizations of plant thioesterases (Harwood, 1996) indicate that the substrate specificities of the acyl-ACP thioesterases may play a major role in determining when the acyl chains are cleaved. Thus this directly influences the pools of free fatty acids available for incorporation into complex glycerolipids. Since there is a relatively high percentage of palmitic acid (22%) in the triacylglycerols (TAGs) of cottonseed oil, we originally speculated that the specific activity of a palmitoyl-ACP thioesterase (PATE) may be responsible for the high level of palmitic acid incorporated into cottonseed storage lipids. Thus the overall goal of my dissertation research was to functionally characterize a cDNA clone encoding a putative FatB acyl-ACP thioesterase from cotton and to evaluate its role in influencing palmitic acid accumulation in cotton.

Although the role of FatB thioesterases has been elucidated in *Arabidopsis* (Dörmann et al., 2000), we tested the current model proposed by Somerville et al. (2000) by investigating the role of FatB thioesterases in a different plant system (cotton, *Gossypium hirsutum* L.). The current model proposes that there are constitutive as well as seed-specific FatB thioesterase isoforms. Based on the results presented in this dissertation, the *FatB* cDNA clone isolated in cotton represents the second constitutive FatB thioesterase characterized (*Arabidopsis FatB* cDNA was the first clone encoding a

constitutive FatB thioesterase to be characterized). The *Arabidopsis* study did not explore the consequences of constitutively overexpressing FatB thioesterases in plants. Our study indicated that overexpressing FatB constitutively influenced the fatty acid compositions of complex glycerolipids and directly impacted plant growth and development (Chapter 3). This work provided the basis for future research to examine the influence of palmitic acid content on plant membrane function. In addition, this work provided data supporting the notion that FatB thioesterses are key targets for metabolic engineering of altered palmitic acid levels in storage oils of cottonseeds.

In chapter one, we explored the existence of an endogenous palmitoyl-ACP thioesterase in cotton. We speculated that an *Arabidopsis* FatB homologue existed in cotton because the *Arabidopsis FatB* cDNA was used as a probe to isolate the cotton cDNA clone. This notion was based on amino acid sequence alignment, which indicated that the cotton preprotein had 63% identity to the *Arabidopsis* FatB preprotein. Thus it was important to provide biochemical and molecular data to support this notion. The preliminary survey of endogenous thioesterase activity and relative transcript abundance in various cotton tissues/organs indicated that acyl-ACP thioesterase activity towards 16:0-ACP was highest in developing embryos of 30-35 dpa. During this developmental stage, oil and protein reserves are accumulating at maximum rates. Because palmitoyl-ACP thioesterase (PATE) activity peaked at this stage, we speculated that PATE may be responsible for the incorporation of palmitic acid into glycerolipids of cotton seed oils. In addition, slot blots revealed that the highest PATE transcript abundance was detected at this developmental stage. Agarose gels of RT-PCR products amplified from total RNA

of developing embryos indicated that the PATE transcript was present in 28-35 dpa embryos. These data suggested that the developmental change in PATE activity was due to a change in PATE gene expression. This study provided the basis for formulating the hypothesis, which states that a palmitoyl-ACP thioesterase plays a role in regulating palmitic acid content in cotton.

Transgenic cotton plants harboring FatB expression constructs were used to elucidate the role of FatB thioesterases in cotton. It was important to first examine if the *FatB* cDNA clone encodes a functional protein product (chapter two). Though there were problems expressing the full-length cotton *FatB* cDNA in *E. coli* cells due to the toxicity of the excess amounts of free fatty acids or the over-accumulation of the recombinant protein (with stromal targeting domains), data presented in chapter two suggested that the cotton FatB cDNA encoded a functional palmitoyl-ACP thioesterase despite its truncated size (the smaller 35 kDa polypeptide product likely represented translation at an internal start site). This 35kDa protein product was shown to be functional *in vivo* and *in vitro*. When acyl-CoA synthetase minus *E. coli* mutants were transformed with the cotton *FatB* cDNA, there was a substantial increase in the release of palmitic acid into the culture medium. Also, in transformed *E. coli* cells there was a preference for 16:0-ACP over 18:1-ACP *in vitro*, indicating that the cDNA clone indeed encodes a member of the FatB class of thioesterases with preference for saturated acyl-ACPs over unsaturated acyl-ACPs (FatA).

In chapter three, I described the production and characterization of transgenic cotton plants harboring the FatB sense and anti-sense expression constructs under the

transcriptional control of the CaMV 35S promoter. The manipulation of FatB expression

resulted in altered palmitic acid content in some cotton tissues, indicating that FatB may

play a direct role in regulating the amount of palmitic acid distributed to various plant

cells. Overexpression of the *FatB* cDNA in transgenic somatic embryos resulted in

elevated levels of palmitic acid (up to 70% of the total). The proportion of palmitic acid

in wild-type zygotic embryos and vector-only control somatic embryos are only about

25% of the total fatty acid composition. These transgenic plants also provided new

insights into the regulation of palmitic acid content during plant growth and development.

The PATE plants, overexpressing FatB, did not develop normally in soil. This is perhaps

due to the change in membrane lipid composition such that the chloroplast membranes

were nonfunctional. Some ASPATE plants, expressing the anti-sense *FatB* cDNA, also

did not development normally. These plants with the "dwarf" phenotype had smaller

leaves, all with smaller epidermal cells (including smaller guard cells) suggesting that a

reduction in palmitic acid may have limited leaf expansion. The data described in

chapter three collectively revealed that the manipulation of FatB thioesterase expression

altered levels of palmitic acid in some cotton tissues indicating that the FatB thioesterase

directly plays a role in regulating palmitic acid levels. Furthermore, this work provides

the basis for future studies of the influence of palmitic acid content on plant membrane

function in other plant systems.

The data described in this dissertation paper altogether support the notion that the

FatB palmitoyl-ACP thioesterase can be a potential target for metabolic engineering

aimed at altering palmitic acid levels in cottonseeds. It has been shown that genetic

manipulation to alter the fatty acid composition of storage TAGs is possible without

detrimental effects to the host plant, thus resulting in the production of novel oils with

improved nutritional characteristics (Kinney, 1994; Harwood, 1996; Kinney, 1997;

Kinney and Knowlton, 1998). The focus of these metabolic engineering projects is to

target selected enzymes of the fatty acid biosynthetic pathway and to examine how they

influence plant lipid accumulation. Two enzymes of plant lipid metabolism have

received the most attention for altering oilseed fatty acid composition. One enzymatic

target is the acyl-ACP thioesterases, which essentially determine the pool of available

fatty acids exported from seed plastids for oil assembly (e.g. the cotton FatB acyl-ACP

thioesterase is described in this dissertation). The other enzymatic target is the fatty acid

desaturases (*fad*), which modify the number of double bonds in fatty acyl chains

incorporated into seed TAGs. Thus it may be possible to manipulate the activities of

acyl-ACP thioesterases and/or fatty acid desaturases to produce novel cottonseed oils,

which include mid-oleic, low-saturate, and high-saturate oils. Specifically targeting the

FatB gene (palmitoyl-ACP thioesterase in cotton), which likely regulates palmitic acid

content (Dörmann et al., 2000), can alter the levels of saturated fatty acids while targeting

the *fad*2 gene will alter the proportion of monounsaturated (primarily 18:1, oleic acid)

fatty acid in cottonseed oil.

Comprehensive References

1. **Anderson MLM, Young BD** (1985) Quantitative Filter Hybridization. *In* Nucleic Acid Hybridization: A Practical Approach. Hames BD, Higgins SJ (eds.) IRL Press, Washington, DC. pp. 73-111.

2. **Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K,** eds. (1987) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York. pp. 2.5.5, 6.0.1-6.3.6.

3. **Benton WD, Davis RW** (1977) Screening λgt recombinant clones by hybridization to single plaques in situ. *Science* 196: 180-182.

4. **Bradford MM** (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 12: 248-254.

5. **Browse JA, McCourt PJ, Somerville CR** (1986) A mutant of *Arabidopsis* deficient in C18:3 and C16:3 leaf lipids. *Plant Physiol* 81: 859-964.

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

7. **Chapman KD, Sprinkle WB** (1996) Developmental, tissue-specific, and environmental factors regulate the biosynthesis of *N*-acylphosphatidylethanolamine in cotton (*Gossypium hirsutum* L.), *J. Plant Physiol.* 149: 277-284.

8. **Chapman KD, Trelease RN** (1991) Acquisition of membrane lipids by differentiating glyoxysomes: role of lipid bodies. *J. Cell Biol.* 115:995-1007.

9. **Christie WW** (1982) *Lipid Analysis*, *2nd ed*. Pergamon Press, New York. pp. 52-54.

10. **Cline K, Henry R** (1996) Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell Dev. Biol.* 12: 1-26.

11. **Dehesh K, Edwards P, Hayes T, Cranmer AM, Fillatti J** (1996) Two novel thioesterases are key determinants of the bimodal distribution of acyl chain length of *Cuphea palustris* seed oil. *Plant Physiol.* 110: 203-210.

12. **Del Vecchio AJ** (1996) High-laurate canola. *Inform* 7: 230-243.

13. **Dörmann P, Kridl J, Ohlrogge JB** (1994) Cloning and expression in *Escherichia coli* of a cDNA coding for the oleoyl-acyl carrier protein thioesterase from coriander (*Coriandrum sativum* L.). *Biochim Biophys Acta* 1212: 134-136.

14. **Dörmann P, Spener F, Ohlrogge JB** (1993) Characterization of two acyl-acyl carrier protein thioesterases from developing *Cuphea* seeds specific for medium-chain and oleoyl-acyl carrier protein. *Planta* 189: 425-432.

15.   **Dörmann P, Voelker TA, Ohlrogge JB** (1995) Cloning and expression in *E. coli* of a novel thioesterase from *Arabidopsis thaliana* specific for long chain acyl-acyl carrier proteins. *Arch Biochem Biophys* 36: 612-618.

16. **Dörmann P, Voelker TA, Ohlrogge JB** (2000) Accumulation of palmitate in *Arabidopsis* mediated by the acyl-acyl carrier protein thioesterase FATB1. *Plant Physiol* 123: 637-643.

17. **Eccleston VS, Cranmer AM, VoelkerTA, Ohlrogge JB** (1996) Medium-chain fatty acid biosynthesis and utilization in *Brassica napus* plants expressing lauroyl-acyl carrier protein thioesterase. *Planta* 198: 46-51.

18. **Eccleston VS, Ohlrogge JB** (1998) Expression of lauroyl-acyl carrier protein thioesterase in *Brassica napus* seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set point for triacylglycerol accumulation. *Plant Cell* 10: 613-622.

19. **Fan J, Ranu RS, Smith C, Ruan C, Fuller CW** (1996) DNA sequencing with [α-^{33}P]-labeled ddNTP terminators: a new approach to DNA sequencing with Thermo Sequenase DNA polymerase. *BioTechniques* 21: 1132-1137.

20. **Feinberg AP, Vogelstein B** (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132: 6-13

21. **Fergusen DL, Turley RB, Meredith WR, Jr.** (1996) Comparison of protein profiles during cotton (*Gossypium hirsutum* L.) fiber cell development with partial sequences of two proteins. *J. Agricultural and Food Chemistry* 44: 4022-4029.

22. **Firoozabady E, DeBoer DL, Merlo DJ, Halk EL, Amerson LN, Rashka KE, Murray EE** (1987) Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. *Plant Mol. Biol.* 10: 105-116.

23. **Gavel Y, von Heijne G** (1990) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett.* 261: 455-458.

24. **Harwood JL** (1988) Fatty acid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39: 101-138.

25. **Harwood JL** (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochim Biophys Acta* 1301: 7-56.

26. **Havaux M** (1987) Effects of chilling on the redox state of the primary electron acceptor Q_A of photosytem II in the chilling-sensitive and resistant plant species. *Plant Physiol Biochem* 25: 735-743.

27. **Hugly S, Somerville C** (1992) A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. *Plant Physiol* 99: 197-202.

28. **Hunt AG** (1994) Messenger RNA 3' end formation in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 45: 47-60.

29. **Hunt T** (1980) The initiation of protein synthesis, *Trends Biochem. Sci.* 5: 178.

30. **Jones A, Davies HM, Voelker TA** (1995) Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. *Plant Cell* 7: 359-371.

31. **Kinney AJ** (1994) Genetic engineering of the storage lipids of plants. *Current Opinion in Biotechnology* 5: 144-151.

32. **Kinney AJ** (1997) Genetic engineering of oilseeds for desired traits. *In* Genetic Engineering, vol 19, Setlow JK (ed.) Plenum Press, New York, pp. 149-166.

33. **Kinney AJ, Knowlton S** (1998) Designer oils: the high oleic soybean. *In* Genetic Modification in the Food Industry, Roller S, Harlander S (eds.) Blackie Academic and Professional, New York, pp. 193-213.

34. **Klein K, Steinberg R, Fiethen B, Overath P** (1971) Fatty acid degradation in *Escherichia coli*: An inducible system for the uptake of fatty acids and further characterization of *old* mutants, *European J. Biochem.* 19: 442-450.

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

36. **Leonard JM, Slabaugh MB, Knapp SJ** (1997) *Cuphea wrightii* thioesterases have unexpected broad specificities on saturated fatty acids. *Plant Mol. Biol.* 34: 669-679.

37. **McCarthy AD, Hardie DG** (1984) Fatty acid synthase - an example of protein evolution by gene fusion. *Trends Biochem. Sci*. 9:60-63.

38. **Merion, M. And Warren, W.** (1989) Purification of supercoiled plasmids from crude cell lysates using high-performance anion exchange chromatography. *BioTechniques* 7: 60-67.

39. **Miguel M, James D, Dooner H, Browse J** (1993) *Arabidopsis* requires polyunsaturated lipids for low temperature survival. *Proc Natl Acad Sci USA* 90: 6208-6212.

40. **Murphy DJ** (1993) Plant Lipids: Their Metabolism, Function, and Utilization. *In* Plant Biochemistry and Molecular Biology. Lea PJ, Leegood RC (eds.) John Wiley and Sons, New York, pp. 113-128.

41. **Ni, W., and Trelease, R.N.** (1991) Two genes encode the two subunits of cottonseed catalase. *Arch. Biochem. Biophys*. 289: 237-243.

42. **Ohlrogge JB** (1982) Fatty acid synthase: plants and bacteria have similar organization. *Trends Biochem. Sci.* 7: 386-387.

43. **Ohlrogge JB, Browse J** (1995) Lipid biosynthesis. *Plant Cell* 7:957-970.

44. **Ohlrogge JB, Jaworski JG** (1997) Regulation of fatty acid synthesis. *Annu Rev Plant Physiol Plant Mol Biol* 48:109-136.

45. **Ohlrogge JB, Jaworski JG, Post-Beittenmiller D** (1993) De novo fatty acid biosynthesis. *In* Moore TS, Jr (ed.) Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 3-32.

46. **Ohlrogge JB, Kuhn DN, Stumpf PF** (1979) Subcellular localization of the acyl carrier protein in leaf protoplasts of *Spinacia oleroacea*. *Proc. Natl. Acad. Sci. USA* 76: 1194-1198.

47. **Overath P, Pauli G, Schairer HU** (1969) Fatty acid degradation in *Escherichia coli*: An inducible acyl-CoA synthetase, the mapping of *old*-mutations, and the isolation of regulatory mutants, *European J. Biochem.* 7: 559-574.

48. **Pappan K, Austin-Brown S, Chapman KD, Wang X** (1998) Substrate selectivities and lipid modulation of plant phospholipase Dα, -β, and -γ, *Arch. Biochem. Biophys.* 353: 31-140.

49. **Paterson AH, Brubaker CL, Wendel JF** (1993) A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. *Plant Mol. Biol. Rep.* 11: 122-127.

50. **Pirtle RM, Yoder DW, Huynh TT, Nampaisansuk M, Pirtle IL, Chapman KD** (1999) Characterization of a palmitoyl-acyl carrier protein thioesterase (FatB1) in cotton. *Plant Cell Physiol* 40: 155-163.

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

52. **Reed KC, Mann DA** (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucl. Acids Res*. 13: 7207-7221.

53. **Routaboul J, Fisher SF, Browse J** (2000) Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiol* 124: 1697-1705.

54. **Sagerstrŏm CG, Sive HL** (1996) RNA Blot Analysis. *In* A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis. Krieg PA (ed.) Wiley-Liss, New York pp. 83-103.

55. **Sambrook J, Fritsch EF, Maniatis T** (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

56. **Schãgger H** (1994) Denaturing electrophoretic techniques, *In*: Von Jagow G., Schãgger H. (eds.), A Practical Guide to Membrane Protein Purification, Academic Press, San Diego, pp. 59-79.

57. **Shanklin J** (2000) Overexpression and purification of the *Escherichia coli* inner membrane enzyme acyl-acyl carrier protein synthase in an active form, *Protein Expression and Purification* 18: 355-360.

58. Shine J, Dalgarno L (1974) The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosomal binding sites, *Proc. Natl. Acad. Sci. USA* 71: 1341-1346.

59. Somerville C, Browse J, Jaworski J, Ohlrogge JB (2000) Lipids. *In* Biochemistry and Molecular Biology of Plants, Buchanan B, Gruissem W, Jones R (eds.) American Society of Plant Physiologists, Rockville, MD, pp 456-527.

60. Speicher D.W (1999) Electrophoresis, *In*: Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT (eds.) Current Protocols in Protein Science, vol.1, John Wiley & Sons, Inc., Seattle, pp. 10.1.1-10.1.34.

61. Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci.* 84: 4767-4771.

62. Thomas JC, Adams DG, Keppenne VD, Wasmann CC, Brown JK, Kanost MR, Bohnert HJ (1995) Protease inhibitors of Maduca sexta expressed in transgenic cotton. *Plant Cell Rep.* 14: 758-762.

63. Trelease RN, Doman DC (1984) Mobilization of oil and wax reserves. *In* Seed Physiology, vol 2, Murray DL (ed.) Academic Press, New York, pp 201-145.

64. Trolinder NL, Goodin JR (1988) Somatic embryogenesis in cotton (*Gossypium*): Requirements for embryo development and plant regeneration. *Plant Cell Tissue Org. Cult.* 12: 43-53.

65. Turley RB, Chapman KD (1998) Ontogeny of cottonseeds: gametogenesis, embryogenesis, germination, and seedling growth. *In* Cotton Physiology - Book II,

Chapter 38. Steward M, Oosterhuis D, Heitholt J (eds.) National Cotton Council, Memphis, TN. In press.

66. **Umbeck P, Johnson G, Baron K, Swain W** (1987) Genetically transformed cotton (*Gossypium hirsutum* L.) plants. *Biotechnology* 5: 263-266.

67. **Voelker T** (1996) Plant acyl-ACP thioesterase: Chain-length determining enzymes in plant fatty acid biosynthesis. *In* Genetic Engineering, vol 18, Setlow JK (ed.) Plenum Press, New York, pp 111-133.

68. **Voelker TA , Davies HM** (1994) Alteration of the specificity and regulation of fatty acid synthesis of *E. coli* by expression of a plant medium-chain acyl-ACP thioesterase, *J. Bacteriol.* 176: 7320-7327.

69. **Voelker TA, Jones A, Cranmer AM, Davies HM, Knutzon DS** (1997) Broad-range and binary-range acyl-acyl carrier protein thioesterases suggest an alternative mechanism for medium-chain production in seeds. *Plant Physiol.* 114: 669-677.

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

71. **Wan CY, Wilkins TA** (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* 223: 7-12.

72. **Wilkins TA, Smart LB** (1996) Isolation of RNA from plant tissue. *In* A Laboratory Guide to RNA Isolation, Analysis and Synthesis. Kreig PA (ed.) Wiley-Liss, New York. pp. 21-41.

73. **Wu J, Lightner J, Warwick N, Browse J** (1997) Low temperature damage and subsequent recovery of *fab*1 mutant *Arabidopsis* exposed to 2°C. *Plant Physiol* 113: 347-356.

74. **Yoder DW, Nampaisansuk M, Pirtle IL, Chapman KD, Pirtle RM** (1999) Molecular cloning and nucleotide sequence of a gene encoding a cotton palmitoyl-acyl carrier protein thioesterase. *Biochim Biophys Acta* 1446: 403-413.

75. **Yuan L, Nelson BA, Caryl G** (1996) The catalytic cysteine and histidine in the plant acyl-acyl carrier protein thioesterases. *J. Biol. Chem*. 271: 3417-3419.