IDENTIFICATION AND QUANTIFICATION OF LIPID METABOLITES IN COTTON FIBERS: RECONCILIATION WITH METABOLIC PATHWAY PREDICTIONS FROM DNA DATABASES.

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Thesis Prepared for the Degree of

MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

May 2004

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Wanjie, Sylvia W., Identification and quantification of lipid metabolites in cotton fibers: Reconciliation with metabolic pathway predictions from DNA databases. Master of Science (Biochemistry), May 2004, 65 pp., 1 table, 11 illustrations, references, 26 titles.

The lipid composition of cotton (*Gossypium hirsutum*, L) fibers was determined. Fatty acid profiles revealed that linolenate and palmitate were the most abundant fatty acids present in fiber cells. Phosphatidylcholine was the predominant lipid class in fiber cells, while phosphatidylethanolamine, phosphatidylinositol and digalactosyldiacylglycerol were also prevalent. An unusually high amount of phosphatidic acid was observed in frozen cotton fibers. Phospholipase D activity assays revealed that this enzyme readily hydrolyzed radioactive phosphatidylcholine into phosphatidic acid. A profile of expressed sequence tags (ESTs) for genes involved in lipid metabolism in cotton fibers was also obtained. This EST profile along with our lipid metabolite data was used to predict lipid metabolic pathways in cotton fiber cells.

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ABBREVIATIONS

AAPT: Aminoalcohol phosphotransferase

ACCase: AcetylCoA Carboxylase

ACP: Acyl carrier protein

ASG: Acylated sterol glycosides

CDP: Cytidine diphosphate

CoA: Coenzyme A

CTP: Cytidine triphosphate

DAG: Diacylglycerol

DAGAT: Diacylglycerol acyltransferase

Dpa: Days post anthesis

DGDG: Digalactosyldiacylglycerol

ESI: Electrospray ionization

ELSD: Evaporative light scattering detection

ESTs: Expressed sequence tags

FAD: Fatty acyl desaturase

FAME: Fatty acid methyl esters

GC: Gas chromatography

G3P: Glycerol-3 phosphate

HMG-CoA: Hydroxymethylglutaryl Coenzyme A

HPLC: High performance liquid chromatographic

KAS: Ketoacyl ACP synthase

LPA: Lysophosphatidic acid

LPAAT: Lysophospahtidic acid acyltransferase

LTP: Lipid transfer proteins

MGDG: Monogalactosyldiacylglycerol

MS/MS: Tandem mass spectrometry

PA: Phosphatidic acid

PC: Phosphatidylcholine

PDAT: Phospholipid diacylglycerol acytransferase

PE: Phosphatidylethanolamine

PG: Phosphatidylglycerol

PI: Phosphatidylinositol

PLD: Phospholipase D

PS: Phospahtidylserine

TAG: Triacylglycerol

TCs: Tentative consensus sequences

TIGR: The Institute for Genomic Research

SG: Sterol glycosides

UNT: University of North Texas

CHAPTER 1

INTRODUCTION

Cotton fibers are trichomes that arise from differentiation of ovular epidermal cells near or on the day of anthesis (Lang, 1938). Development of these cells progresses through stages of elongation and secondary wall deposition forming extremely long single cells, over an inch long (Basra, 1984) during a period of about 25 days. This period of rapid cellular elongation requires substantial synthesis of macromolecules, such as lipids necessary for the developing vacuole and plasma membranes. The major focus on cotton fiber development has been on cellulose synthesis, which accounts for the bulk of the mass of fiber cells, however lipid metabolism may be an important factor in fiber development.

Electron microscopy studies have shown that fiber initiation occurs near or on the day of anthesis, as fibers emerge as protrusions from the epidermal layer of cotton ovules (Ramsey and Berlin, 1976, Stewart, 1975). This is followed by elongation, in which the cell undergoes rapid expansion with increases in length and diameter, as well as primary cell wall formation. On average this continues for 15 to 27 days (Ramsey and Berlin,1976). Secondary cell wall synthesis begins around 16 to 19 days post anthesis (dpa) and is thought to overlap with elongation (Schubert et al., 1973). This process consists of the deposition of successive layers of cellulose microfibrils in a helical pattern around fiber cells (Basra, 1984, Kim and Triplett, 2001). Maturation of fibers ends about 45 to 60dpa with seed capsule dehiscion, dehydration, and collapse of fiber cells (Basra and Malik 1984).

Membrane Lipid Synthesis

Synthesis of cellular compartments, including lipids for incorporation into the plasma membrane, is necessary during the period of rapid cell expansion in cotton fiber cells. Fatty acid biosynthesis in plants occurs de novo in plastids and several enzymes are involved in this process (See Chapter 3 and Figure 11 A). Fatty acids synthesized in the plastid can either be incorporated into plastidial glycerolipids or esterified to CoA and transported to the ER to be incorporated into glycerolipids synthesized in this compartment. Glycerolipid synthesis in plastids or the ER begins with transfer of two fatty acyl chains onto glycerol-3 phosphate, forming phosphatidic acid (PA) which serves as a precursor for synthesis of most glycerolipids. (For glycerolipid synthesis see Chapter 3 and Figure 11 B and 11C).

Plant membrane lipids mainly consist of glycerolipids, as well as a small percent of sphingolipids (Somerville et al., 2000). The lipid composition of membranes of different plant cells varies with each having a distinct glycerolipid composition and each lipid class having a distinct fatty acid composition (Wallis and Browse, 2002). Structural membrane glycerolipids mainly contain the common16-carbon or 18-carbon fatty acids, which may be unsaturated with up to three double bonds (Millar et al., 2000) whereas large number of unusual fatty acids have been found in seed oil acyl chains Voelker, 2001, Millar et al., 2000). Exclusion of unusual fatty acids from membrane lipids has been attributed to the physical and chemical properties of these fatty acids, which are thought to be incompatible with membrane bilayer integrity (Millar et al., 2000). Most membranes consist of about 50% of polyunsaturated acyl lipids and the composition of these lipids is critical for growth and development (Wallis and Browse, 2002).

Determining the lipid profiles of cotton fiber cells may be important in understanding their role in the development of these unusually elongated cells.

The lipid content in mature cotton fibers has been found to be about 0.5%, and includes saturated and unsaturated hydrocarbons, waxy esters, glycerides, long chain alcohols, sterols, glycols and aliphatic alcohols (Amin and Truter, 1972). Developing fiber cells undergoing rapid elongation, have a higher lipid content and have been found to incorporate the majority of ^{14}C labeled acetate into polar lipids, at a rate that declines as cell elongation ceases from10 to 20dpa (Basra, 1984). As polar lipids are the major constituents of lipid bilayers, these results suggest that the cell is engaged in active membrane synthesis during early stages of development.

Modern techniques, which allow for specific classification, can now be used to analyze lipid composition. Electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) is currently used to determine the classes, molecular species, and amount of lipids in plant cells (Welti et al. , 2002). This technique was applied to study the classes and molecular species of polar lipids in developing cotton fiber. High performance liquid chromatographic (HPLC) analysis combined with evaporative light scattering detection (ELSD) or MS is also frequently used to study the composition of lipids especially those containing sterols, sterol esters and sterol glycosides, as better sensitivity is achieved with ELSD than with UV detectors (Christie, 1992). Sterol glycosides and their acylated components in cotton fiber cells, have been implicated as primers for elongation of β1-4 glucan chains which form cellulose microfibrils during secondary cell wall development (Peng et al., 2002), hence lipid extracts were analyzed using ELSD to determine the glycerolipid as well as the sterol composition in cotton fiber cells.

Expressed Sequence Tags in Developing Cotton Fibers

Several genes that are preferentially expressed in cotton fiber during the rapid elongation process have already been identified and include some involved in lipid metabolism, such as those encoding a putative acyltransferase and a putative 24-sterol-C methyltransferase (Chun-Hong, 2002). Transcripts for a gene encoding a fiber specific acyl carrier protein (ACP) were highly expressed during the stage of elongation, and this ACP was thought to play a role in synthesis of membrane lipids (Song and Allen, 1997). Cotton fiber specific cDNAs encoding lipid transfer proteins (LTPs) have been isolated, and shown to have a temporal expression pattern, accumulating from 6dpa to 14dpa (Orford and Timmis, 1997) and transcript levels for one isoform Ltp3 reached a maximum at 15dpa (Ma et al., 1995). The results in these studies indicate that transcription of genes involved in lipid metabolism in cotton fiber occurs in a developmentally regulated manner. Studying the expression of genes in cotton fiber cells during the stage of rapid elongation would provide an insight into the mechanisms responsible for the development of these cells.

More than 50,000 expressed sequence tags (ESTs) from Gossypium sp. fiber have been sequenced by several laboratories (predominantly Brookhaven National Laboratory and Clemson University Genomics Institute) and compiled by The Institute for Genomic Research (TIGR) at the Website at http://www.tigr.org. Most all of these are derived from cotton fibers at 6dpa or 7 to10dpa, during the stage of rapid cell elongation, and most likely include expressed genes of lipid metabolism important for this development stage. The relative abundance of ESTs for enzymes involved in lipid metabolism can be used to begin to predict which metabolic pathways are operating

during this stage of rapid cell elongation. The combination of metabolic profiles and DNA database information provides a unique perspective on membrane biogenesis for cell elongation and will serve as a basis for the development of hypotheses regarding the regulation of membrane biogenesis in this important agricultural plant.

Research Objectives

The main objective of this research was to investigate the role of lipids in rapidly elongating cotton fiber cells. We analyzed the lipid composition of cotton fibers at different stages of development, which may be crucial in understanding the process of fiber development and plant elongation in general. The second objective was to estimate a gene expression profile for lipid metabolism genes in cotton fibers. Towards this end we compiled a list of expressed sequence tags (ESTs) for enzymes involved in lipid metabolism in developing cotton fibers, using EST data obtained The Institute for Genomic Research (TIGR) through the Website at http://www.tigr.org. The data on EST profiles was viewed in light of the biochemical data and was used to predict lipid metabolic pathways in developing cotton fiber cells.

CHAPTER 2

MATERIALS AND METHODS

Plant Growth and Collection of Cotton Fibers

Cotton (*Gossypium hirsutum*, L. cv Coker 312) plants were greenhouse grown with day/night temperatures of 85 ^{o} to 96 ^{o} F with light and dark cycle of 16/8 hours. Flowering plants were tagged on the day of anthesis, and bolls were harvested at 7 to 10 days post anthesis (dpa), 14dpa, 21dpa and 28dpa. The bolls were placed in a plastic freezer bag and transported on dry ice from the greenhouse to the laboratory, and stored at -80^{o}C if lipid extraction was not performed immediately. Alternatively, to obtain fresh fiber extracts, the whole plant was transported from the greenhouse to the laboratory, and bolls harvested and processed without freezing. Cotton (*Gossypium hirsutum,* L cv Sg747) bolls were kindly provided by Jodi Scheffler (Stoneville MS). Ovules were excised from the bolls and fibers pulled off the ovules, and weighed. Fibers were frozen in liquid nitrogen and crushed into powder using a mortar and pestle. Fibers from maturing bolls were initially chopped into small piece using forceps and a sterile blade prior to freezing in liquid nitrogen. Lipids were then extracted from the crushed fiber suing the method described below. Replicate fiber samples were also obtained and oven dried overnight at 50^{0}C to determine dry weight of our fiber samples.

Chemicals for Lipid Extraction and Transesterification

L-α-phosphatidylcholine, diheptadecanoyl (c17:0) was obtained from Sigma Chemical co, MO and all other chemicals were from Fisher Scientific, PA unless otherwise stated.

Lipid Extraction

Total lipid extraction was by a modified version of the Bligh /Dyer method using isopropanol and chloroform (as described in Chapman and Moore 1993). 0.8 to1g of fiber cells was placed in 2ml hot isopropanol. L-α-phosphatidylcholine, diheptadecanoyl (C17:0) (5 to 10 mg) was added to samples as a quantitative standard and the tubes were incubated at 70^{O}C for 30 minutes. The samples were allowed to cool at room temperature and 1ml of chloroform was then added and the samples allowed to extract overnight at 4^{0}C. Following overnight extraction, phase separation was achieved by addition of 2ml of KCL and 1ml of chloroform, and facilitated by centrifugation at 200xg for 5 minutes. The upper aqueous phase was aspirated off and the remaining chloroform layer was washed two more times with 2ml 1M KCl and for a final wash with 1ml ultrapure water. The chloroform was then evaporated off using a Multivap118 nitrogen evaporator (Organomation Associates, Berlin MA), and 2ml of fresh chloroform was added to the remaining lipid extract. The lipid samples were placed in a teflon lined vial and stored under nitrogen at –20 ^{O}C.

Transesterification

The lipid extracts were evaporated to dryness with nitrogen gas as described above. Transesterification was achieved by addition of 1ml acidic (1% H_2SO_4) methanol and 0.5ml tetrahydrofuran to the samples, which were then incubated for 30 minutes at 65 ^{O}C. Following transesterification, the samples were allowed to cool and 1ml of 5% NaCl and 2 to 3 ml of Hexane were added, and the samples were vortexed thoroughly, then allowed to stand to allow phase separation. The upper hexane layer was then

collected and placed in a clean tube. Hexane was once again added to the remaining aqueous layer and the collection repeated. Finally, the hexane was evaporated off under a gentle stream of nitrogen and the lipid extracts were dissolved in an appropriate volume of hexane for gas chromatographic analysis.

Gas Chromatographic Analysis of Fatty Acid Methyl Esters

Gas chromatographic (GC) analysis of fatty acid methyl esters (FAMEs) was performed using a Hewlett Packard Series II plus 5890 Gas Chromatograph. 1.5 µl to 2.5 µl of sample was injected into a 30 meter SUPERCOWAX^{TM}-10 fused silica capillary column. (Supelco, Bellefonte, PA) at an oven temperature of 200^{O}C with nitrogen as the carrier gas and flame ionization detection was used to quantify fatty acid methyl esters. Identification of fatty acids in samples was achieved by comparison to lipid standards, (FAM E Mix GLC –10; Supelco, Bellefonte, PA), and the amount of each fatty acid was calculated based on the amount of the internal standard.

Analysis of Polar Lipid Classes and Molecular Species

High performance liquid chromatographic (HPLC) analysis of lipid extracts using evaporative light scattering detection (ELSD) and mass spectrometry (MS) was kindly provided by Dr Robert A. Moreau at ARS-USDA Eastern Regional Center, Philadelphia PA. The polar lipid classes and molecular species of our lipid extracts were determined by electrospray ionization (ESI) and tandem mass spectrometry (MS/MS), kindly provided by Dr Ruth Welti, at the Lipidomics center at Kansas State University, Manhattan, KS.

Chemicals for Phospholipase D Activity Assays

L-α- phosphatidylcholine dipalmitoyl, [dipalmitoyl-1-^{14}C] was purchased from NEN Life Sciences, (Boston,MA). L-α- phosphatidylcholine Type II from soybean, cabbage PLD Type V and bovine serum albumin were from Sigma Chemical Co, Mo. All other reagents were purchased from Fisher Scientific, PA unless otherwise stated.

Homogenization of Fiber Cells for PLD assays

Ovules were excised from bolls at different stages of development and the fibers pulled off the surface of the ovules and weighed. The fibers were then ground with a mortar and pestle on ice in homogenization buffer (10mM KCl, 1mM EDTA, 1mM $MgCl_2$, 400mM sucrose, 1mM EGTA, 100mM Kphos buffer pH 7.2). The homogenate was filtered through 2 layers of cheesecloth, and the final volume recorded. Samples were centrifuged for 20 minutes at 10,000xg at 4^0C. The supernatant was collected and protein content of these crude extracts was estimated.

Estimation of Protein Content

The protein content of our crude extracts was estimated by the Bradford assay (Bradford, 1979) using BSA as a protein standard. Briefly, different volumes (5µl, 10µl, 15µl, 20µl) of the fiber extracts were obtained and each mixed with water to a final volume of 50µl, to which 50µl of NaOH and 1ml of Bradford reagent was added, and then vortexed thoroughly. Samples were incubated for 15 minutes at room temperature, then vortexed again and the absorbance at 595nm of each sample was obtained using

a spectrophotometer (Milton Roy company, NY). A BSA standard curve was used to determine the protein content based on the absorbance of each sample.

PLD Activity Assays

PLD activity was assayed as hydrolysis and transphosphatidylation of phoshatidylcholine using L-α- phosphatidylcholine dipalmitoyl, [dipalmitoyl-1-^{14}C] (^{14}C PC) and unlabelled phosphatidylcholine as a substrate. The assay mix consisted of 25µl of 4X assay buffer (100mM MES, 25mM $CaCl_2$ and 0.5mM SDS) and 25µl of lipid master mix (0.4mMol PC and 0.05Ci ^{14}C PC). The lipid master mix was prepared in advance, dried under nitrogen and resuspended in 10ul of ultrapure water with constant vortexing and sonication to resuspend lipid vesicles. The reaction was started by addition of 7µg of protein from crude fiber homogenate in 50µl volume, adjusted with ultrapure water. 95% ethanol (1.5µl) was added to each reaction immediately after the reaction begun. Samples were incubated at 30^{0}C in a water bath, with shaking at 70rpm, for 30 minutes. The reaction was stopped by addition of hot (70^{0}C) isopropanol, incubation at 70^{0}C for 30 minutes. Samples were then cooled for 5 minutes and 1ml of chloroform and 700µl of ultrapure water was added. Lipid extraction was as previously described above. The lipids were later resuspended in 40µl of chloroform and analyzed by thin layer chromatography and radiometric scanning.

Thin Layer Chromatography

One dimensional thin layer chromatography (TLC) was used to separate lipid products of the PLD assays. The lipid samples were applied 4 X 10 µl to 20 X 20cm silica gel

plates (Whatman, layer thickness 250 μm). Phospahtidylcholine and phospahtidylethanol were separated by TLC in a chloroform: methanol: ammonium hydroxide (65:35:5) solvent system for approximately 90 minutes. The TLC plate was then analyzed using a radiometric scanner (Bioscan 200 Imaging Scanner, Bioscan DC), and radiolabelled products quantified as a percentage of the total radioactivity. The radiospecific activity of the substrate was then used to calculate the enzyme activity, which was expressed as mol/min/mg protein.

CHAPTER 3

RESULTS

Fatty Acid Composition of Cotton Fiber Cells

Total lipid extracts were obtained from cotton fiber cells (*Gossypium hirsutum*, L cv Sg747 and Coker 312) harvested at different ages (7, 14, 21 and 28dpa), representative of different stages of fiber development. Fatty acid composition was determined by gas chromatographic (GC) analysis of fatty acid methyl esters (FAMEs), with heptadecanoic acid (17:0) as a quantitative internal standard. The percent by weight of each of the common fatty acids, palmitate (16:0), stearate(18:0), oleate(18:1), linoleate(18:2) and linolenate(18:3) remained relatively constant over the entire period of fiber development (Figure 1). Linolenate was found to be the most abundant fatty acid, accounting for about 40 to over 50% by weight of total fatty acids and palmitate was also fairly prevalant making up 25% to 35% by weight of total fatty acids. Linoleate comprised10% to 15% by weight of fatty acids present, while low levels of oleate and even lower levels of stearate were observed in all stages of development. A small increase in the ratio of saturated to unsaturated fatty acids, was observed in the later stages of fiber development.

The amount of fatty acid per gram of fiber dry weight was determined as well. In Coker 312 fibers the fatty acid content per gram fiber dry weight was highest at 7dpa, while in Sg747 fibers a steady increase was observed from 7dpa to 14dpa. This pattern was typical for individual fatty acids with the exception of 18:1 in Sg747, which remained constant from 7 to 14dpa, and 18:2 in Coker 312, the levels of which were highest at

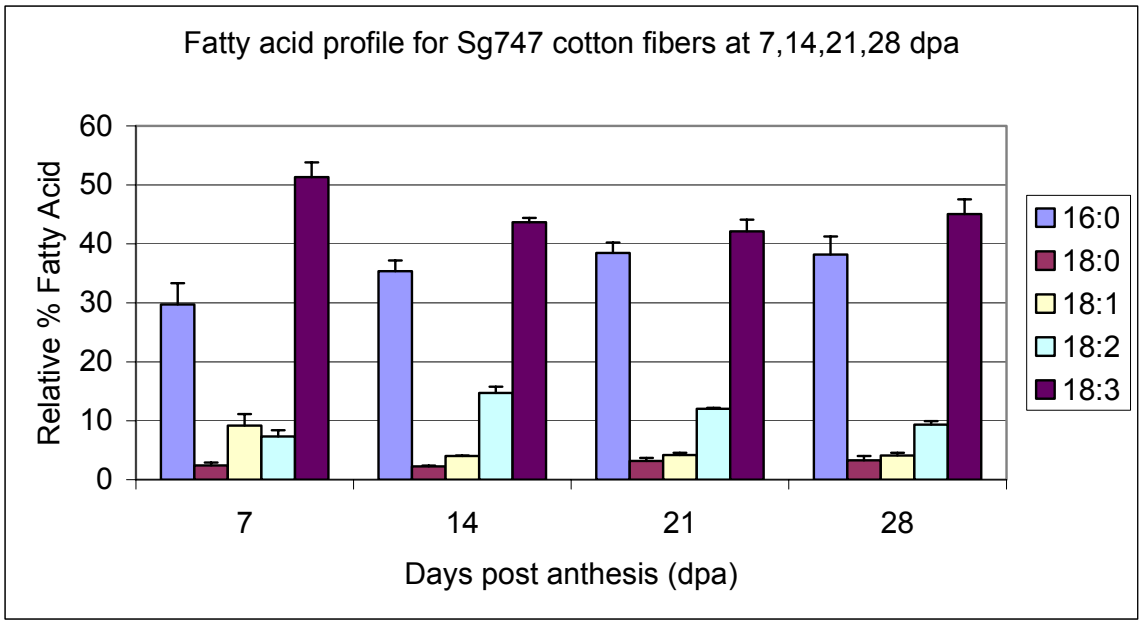
14dpa. A decrease in amount of fatty acid per gram fiber dry weight is observed in later stages of development in fibers from both cultivars.

An increase in total amount of fatty acid per boll was observed going from 7dpa to 21dpa, but the level of fatty acid declined at 28dpa (Figure 3), despite a relative increase in the size of bolls and fiber content. In Sg747 fibers total fatty acid content of increased with age as shown by the fatty acid amounts of 7dpa bolls compared to that of 14dpa bolls. The fatty acid content of fiber cells from bolls harvested 21dpa was similar to that in fiber cells from14dpa bolls, but declined in 28dpa bolls, despite an increase in fiber dry weight over these periods. In Coker 312 fibers a steady increase in total fatty acid amounts was observed from 7dpa to 21dpa followed by a sharp decrease in 28dpa fibers.

The above results may be explained based on what is known about the development of cotton fiber cells. During the early stages of fiber development, cells are undergoing rapid elongation (Basra and Malik, 1984), and are engaged in lipid synthesis for membrane development, hence, an increase in fiber mass is associated with a concomitant increase in lipid amounts. In maturing bolls, fiber cells are involved in secondary cell wall synthesis, which begins around 16dpa to19dpa and continues up to 25dpa (Basra and Mali, 1984). During this stage of development, cellulose is accumulated and accounts for the bulk of the mass in fiber cells. As a result the proportion of lipids relative to the overall weight of fiber is much lower. This would explain, the observed decrease in fatty acid amounts per dry weight in 21dpa fibers, despite an increase in boll size and total fatty acid content. The decrease in total fatty acid amount per boll at 28dpa may be accounted for by a decline in membrane lipid

synthesis in maturing fibers relative to elongating fibers at earlier stages. Cotton embryos begin to synthesize seed oil at around 20-25dpa hence membrane lipid synthesis in fiber cells may decline during this period of triacylglycerol accumulation, in the overall balance of cottonseed development.

A.


Fatty acid profile for Sg747 cotton fibers at 7,14,21,28 dpa
Relative % Fatty Acid
60
50
40
30
20
10
0
7
14
21
28
Days post anthesis (dpa)
16:0
18:0
18:1
18:2
18:3

B.

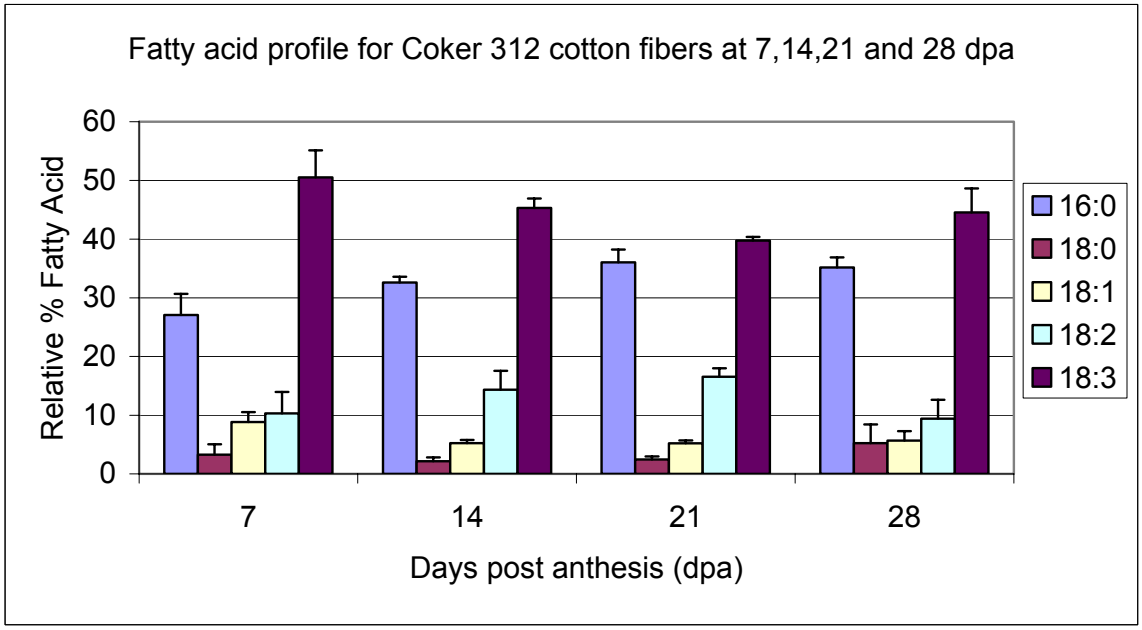
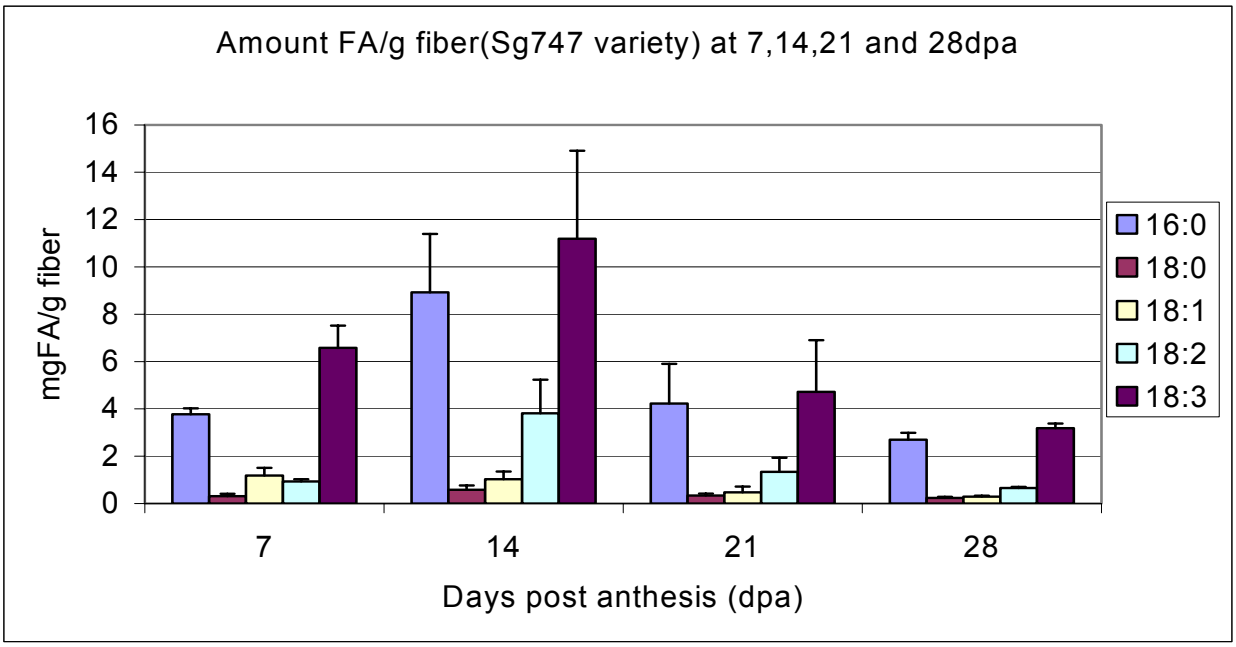

Fatty acid profile for Coker 312 cotton fibers at 7,14,21 and 28 dpa
Relative % Fatty Acid
60
50
40
30
20
10
0
7
14
21
28
Days post anthesis (dpa)
16:0
18:0
18:1
18:2
18:3

Figure 1: The fatty acid composition of cotton *(Gossypium hirsutum* ,L cv Sg747 and Coker 312.) fiber cells at different stages of development (7,14, 21 and 28dpa). Total lipid extracts were obtained from fibers by a modified version of the Bligh and Dyer method, with addition of L-α-phosphatidylcholine, diheptadecanoyl (C17:0) as an internal standard. Following acid catalyzed transesterification, FAMEs were analyzed by gas chromatography and quantified based on the amount of heptadecanoic acid (17:0) in the internal standard. The relative proportions of the common fatty acids present in A. Sg747 fibers and B. Coker 312 fibers, is shown. The overall composition was similar throughout the entire period of fiber growth and 18:3 and 16:0 were the most abundant fatty acids at each stage of development. Bars represent the means and SD of three to four independent extractions.

A.


Amount FA/g fiber(Sg747 variety) at 7,14,21 and 28dpa
mgFA/g fiber
16
14
12
10
8
6
4
2
0
7
14
21
28
Days post anthesis (dpa)
16:0
18:0
18:1
18:2
18:3

B.

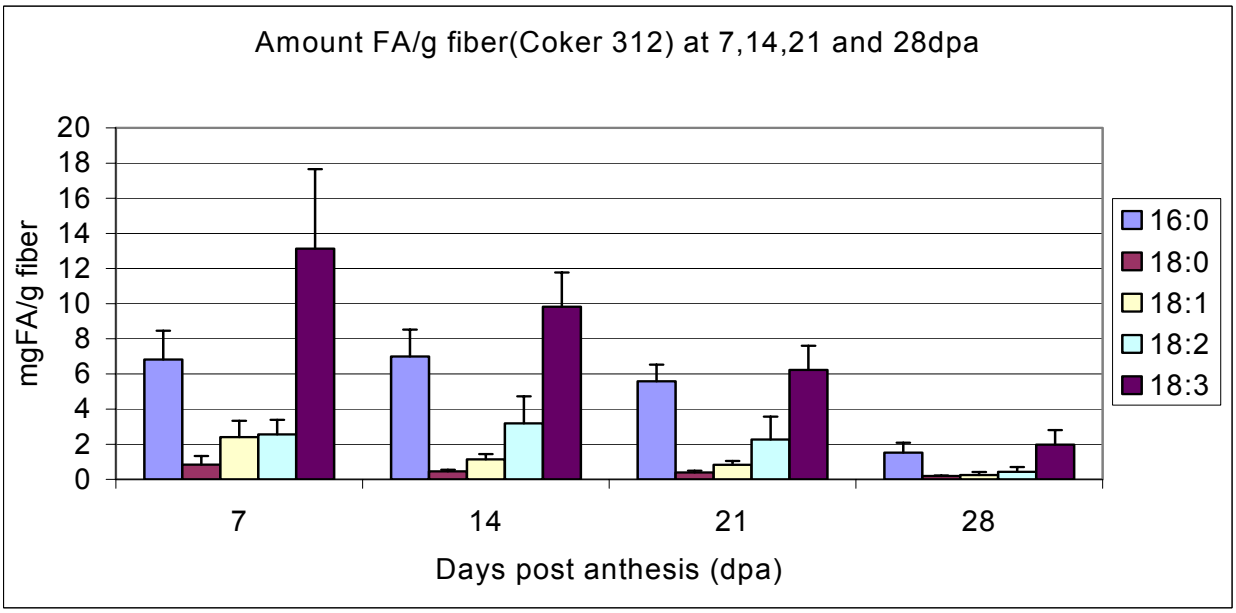

Amount FA/g fiber(Coker 312) at 7,14,21 and 28dpa
mgFA/g fiber
20
18
16
14
12
10
8
6
4
2
0
7
14
21
28
Days post anthesis (dpa)
16:0
18:0
18:1
18:2
18:3

Figure 2: Quantification of fatty acids in cotton fibers per gram dry weight. During the period of rapid cell elongation (7dpa to 14dpa) the proportion of fatty acids relative to the overall mass of fibers appeared to be much higher than that present in the later stages of development. A. In Sg747, the amount of fatty acid per dry weight fiber peaked at 14dpa, and declined thereafter. B .In Coker 312, fatty acid levels were highest around 7dpa, and steadily decreased thereafter. Bars represent the means and SD of three to four independent extractions.

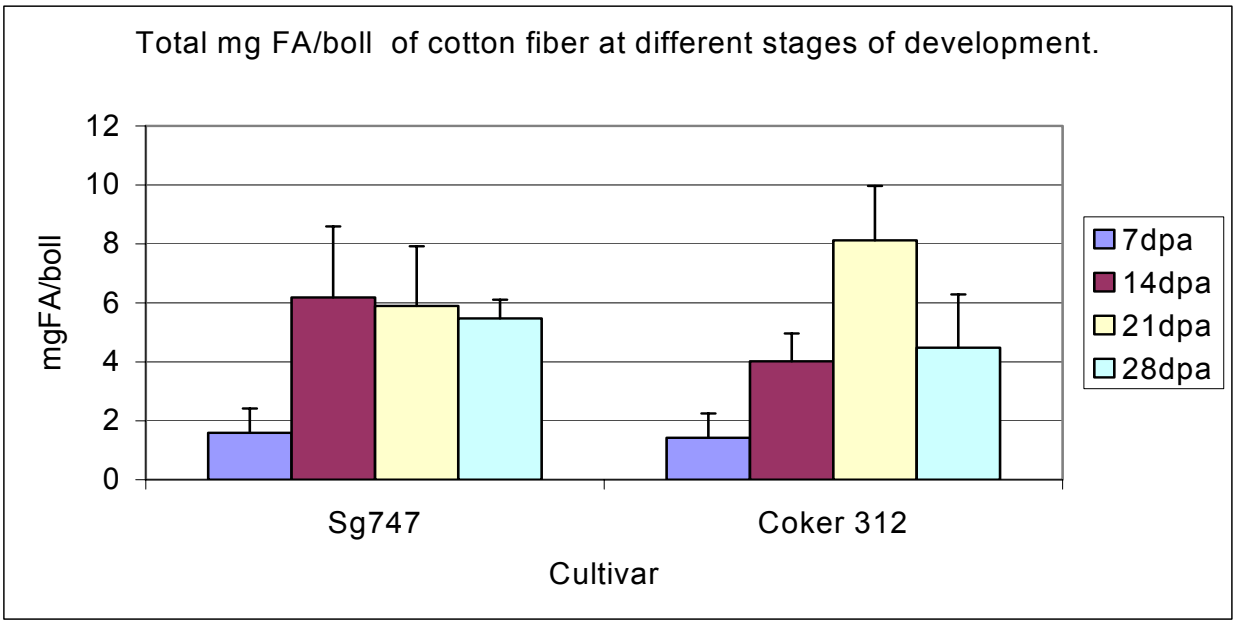


Figure 3: The total amount of fatty acid (mg per boll of fiber) at the different stages of development. A significant increase in the relative amount of fatty acids occurs from 7dpa to 21dpa, while a decrease in total fatty acids is evident from 21dpa to 28dpa, despite an increase in the size of bolls. In Sg747, an increase in total fatty acid content of fibers occurred from 7dpa to 14dpa, and levels seemed to remain constant up to about 21dpa. A decrease in the total amount of lipid was evident at 28dpa, despite an increase in boll size and fiber content. This decrease at 28dpa is more apparent in Coker 312, which showed a steady increase in total fatty acid content of fibers from 7dpa to 21dpa. Bars represent means and SD for three to four independent lipid extractions.

Profiles of Polar Lipids in Cotton Fiber Cells

Polar lipid profiles of elongating cotton fiber cells (7 to10dpa) and maturing cotton fiber cells (21dpa) were determined by HPLC analysis. In elongating fiber cells, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA), were the predominant polar lipid classes comprising 25%, 15%, 40% and 17% by weight of total lipids, respectively (Figure 4). In maturing fiber cells an abundance of PA (43%) was observed, whereas levels of PC (15%) were much lower. The levels of PC and PA varied greatly among individual samples, in both elongating and maturing fiber cells. The proportion of PE (12%) was similar to that in elongating fiber, while PI levels (26%) were much lower. The amount of digalactosyldiacylglycerol (DGDG) was found to be slightly higher in maturing fiber cell, as was the ratio of acylated sterol glycosides (ASG) to sterol glycosides (SG). Such an abundance of PA (17% and 43% by weight of total polar lipid, in elongating and maturing fiber, respectively) was unusual for membrane lipids, and may be attributed to phospholipase D (PLD) mediated hydrolysis of the major phospholipids that can occur during tissue processing and lipid extraction. However, we used a modification of the typical Bligh and Dyer method for lipid extraction, which substitutes 2-propanol for methanol and minimizes the activation of PLD. Additionally, analysis of cotton fiber lipid extracts by ESI followed by tandem MS, in which 17:0 PC was added as internal standard during the lipid extraction process, showed no production of 17:0 PA, whereas large amounts of other PA species, were observed (Figure 5). These results indicate that PA production did not occur during the lipid extraction, but we cannot rule out the possibility that that PA was generated from other glycerolipids during tissue harvesting.

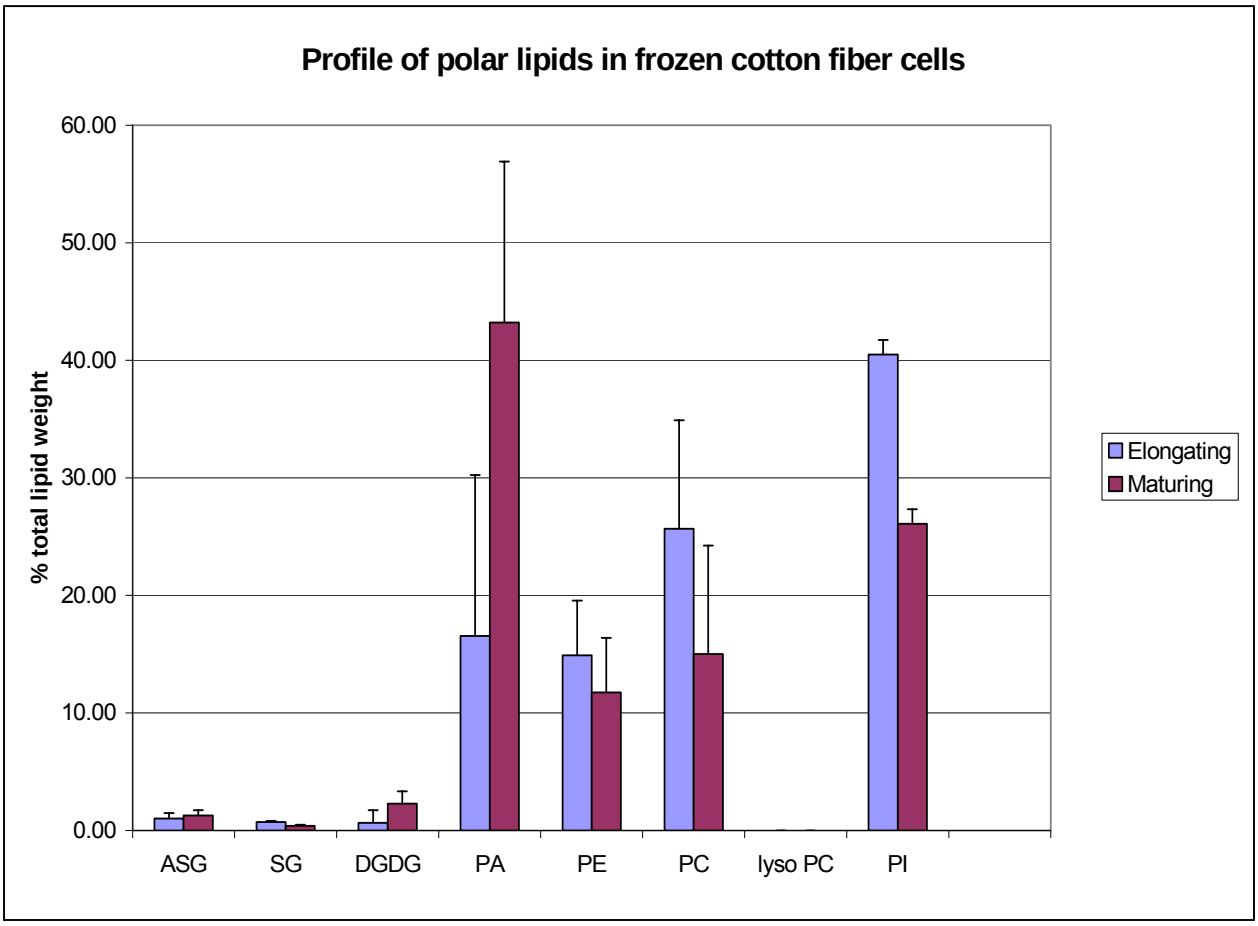
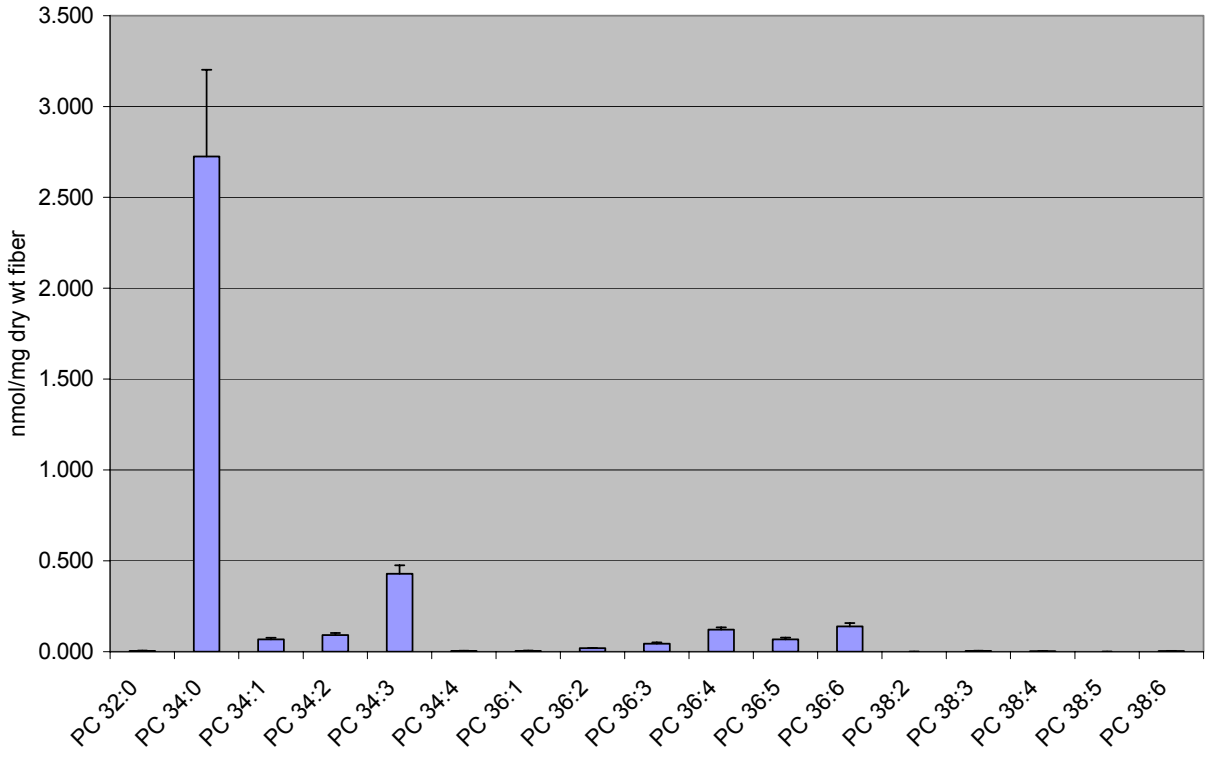


Figure 4: Lipid profiles of cotton fiber cells from frozen elongating bolls and maturing bolls, determined by HPLC analysis. The mean levels of PC and PI were much higher in elongating fiber as compared to maturing fiber, whereas similar levels of PE were observed. Considerable variability was observed in the levels of PC and PA from sample to sample as indicated by the large experimental error. The mean levels of PA were unusually high for membrane lipids. Low levels of DGDG, ASG and SG were observed in both elongating and maturing fiber lipid extracts. Tissue harvest and lipid extractions were performed at UNT and Dr Robert A. Moreau, USDA-ARS Eastern Regional Research Center, Philadelphia, PA performed the HPLC analysis. Bars represent the means and SD for three independent extractions.

PC in Sg747 cotton fiber cells



PA in Sg747 cotton fiber cells

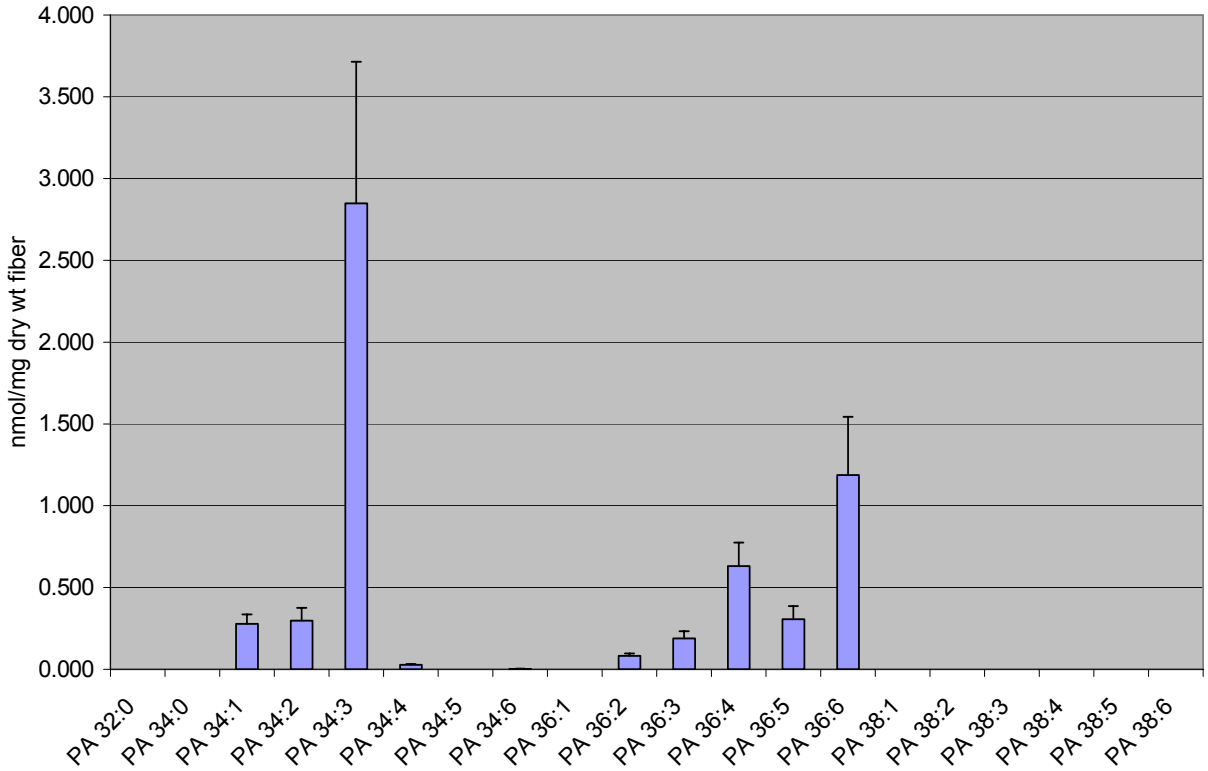
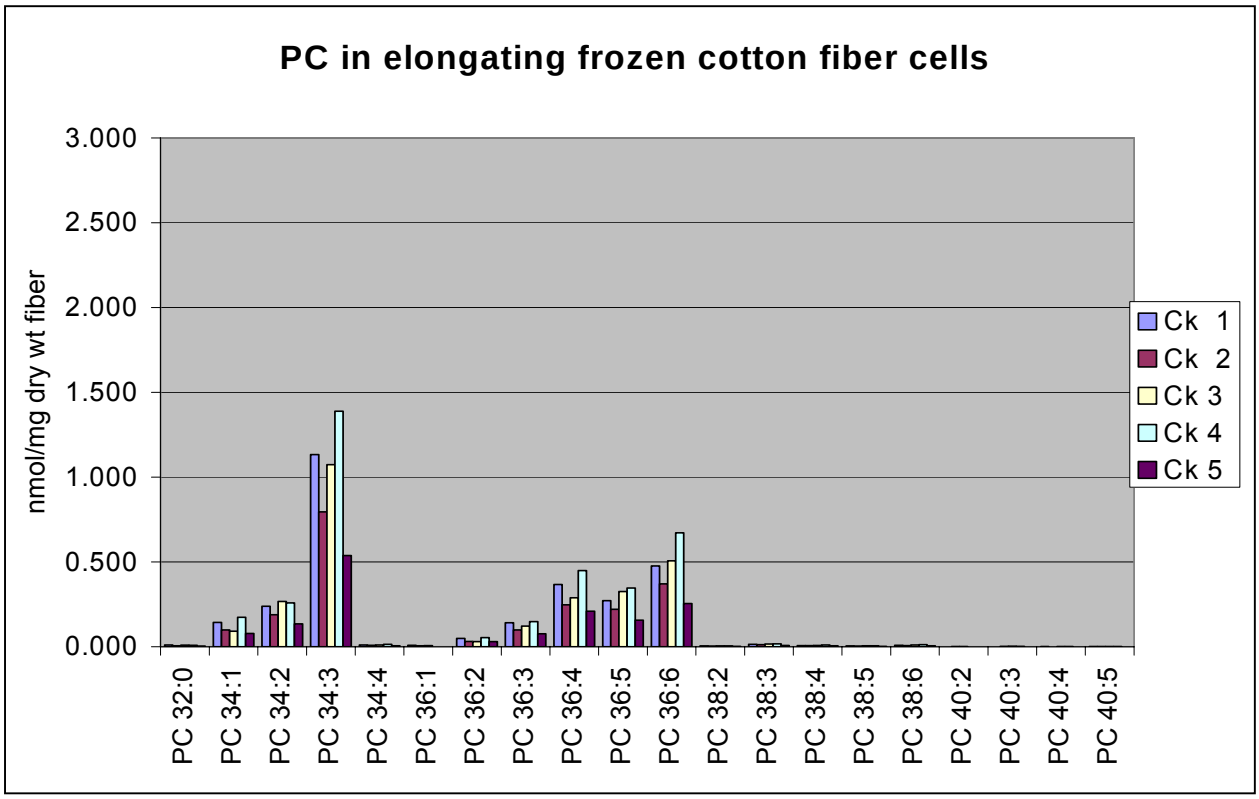
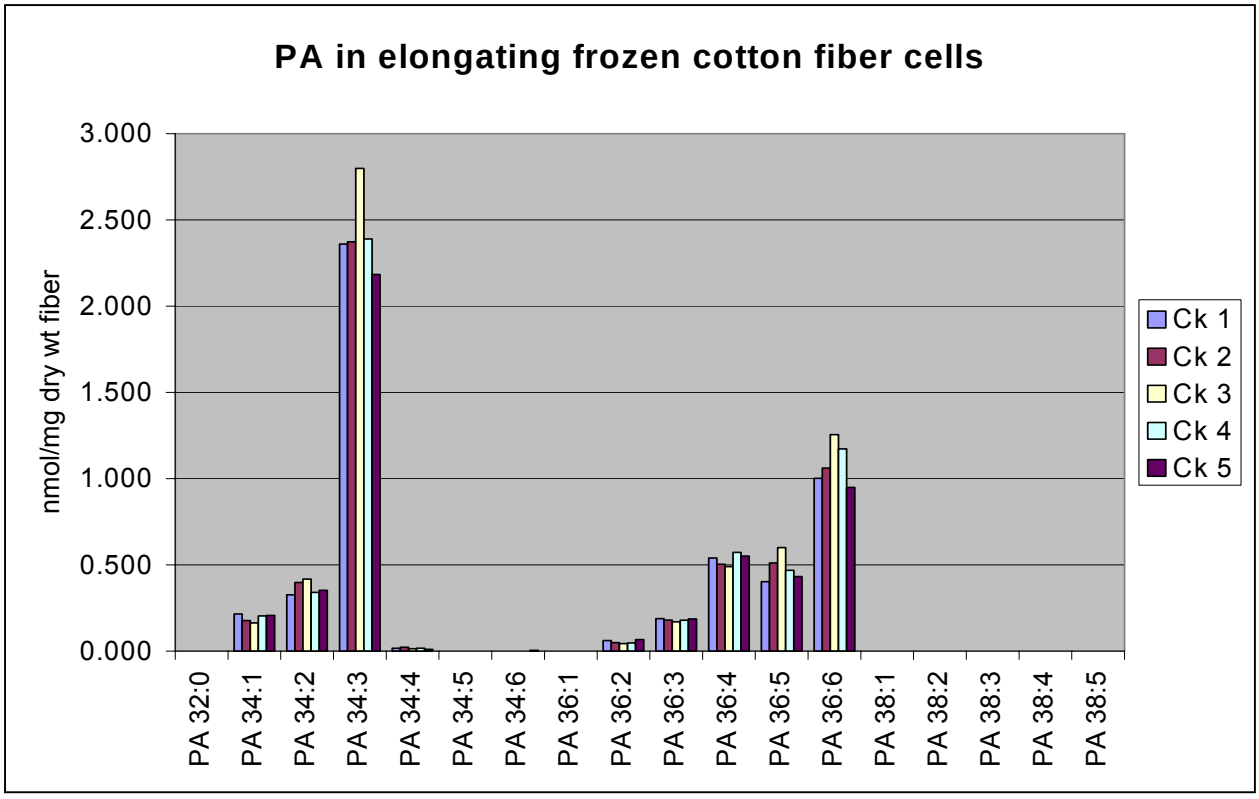


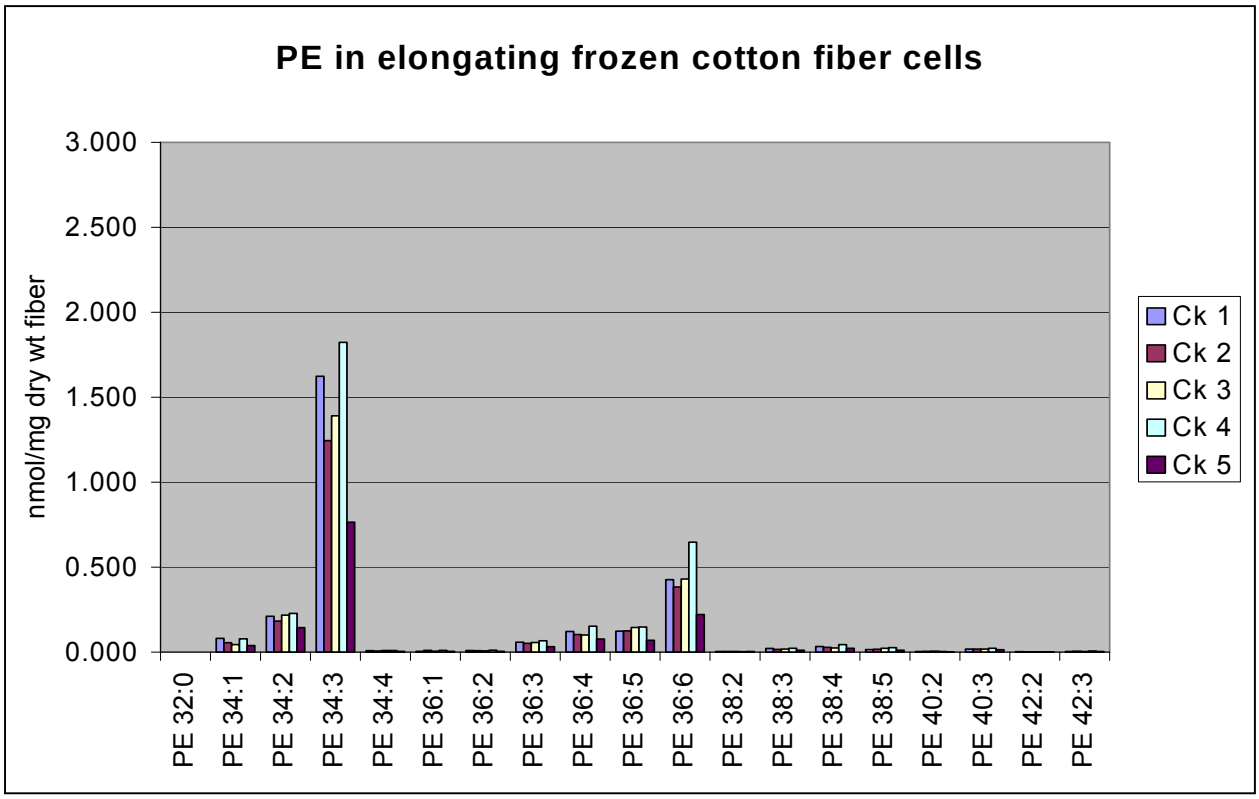
Figure 5: The molecular composition of PC and PA in Sg747 frozen cotton fiber cells. Diheptadecanoyl PC was added as an internal standard, during the lipid extraction process and ESI followed by tandem MS analysis of lipid extracts, revealed that PC 34:0(17:0,17:0) was the most abundant lipid. PC 34:3, and PC 36:6 were also found to be prevalent. An abundance of PA 34:3, and PA 36:6 was also observed. The absence of any PA 34:0, suggests that PLD mediated hydrolysis of PC to PA does not occur during the lipid extraction process. The Sg747 cotton bolls were provided by Jodi Scheffler (Stoneville, MS). Lipid extraction was done at UNT and ESI and tandem MS analysis was performed by Dr Ruth Welti at the Kansas State University Lipidomics Center, Manhattan, Kansas. The bars represent the average of 5 individual lipid extracts.

The classes and molecular species of polar lipids in developing and mature cotton fiber (*Gossypium, hirsutum* L., cv Coker 312 and Acala) were determined by ESI followed by tandem MS. The composition and relative proportions of the different molecular species in each class was found to be similar in developing fiber cells as compared to maturing fiber cells, and the only major difference observed was in the relative amounts of lipid per dry weight. As noted before, maturing fiber is largely engaged in cellulose accumulation and an increase in fiber mass, results in a decrease in lipid per weight fiber. The lipid profiles obtained for frozen Acala fibers were very similar to those of frozen Coker 312 fibers, hence, while the data set presented is for Coker 312 fibers, the inferences drawn here may be extended to other fiber varieties

The major membrane phospholipids, PC, PE and PI, along with PA, were the predominant classes in frozen fiber lipids extracts analyzed (Figure 6). The levels of PA exceeded those of the other membrane lipids, which were present in relatively similar quantities. The most abundant molecular species for PC, PE, PI as well as PA was 34:3(18:3,16:0) and 36:6 (18:3,18:3), was also fairly prevalent. The composition and relative proportions of molecular species found in PA, were similar to those found in PC, PE and PI, suggesting that PA may have been generated by hydrolysis of the other major membrane phospholipids. Other molecular species present included 36:5(18:3,18:2), 36:4(18:3, 18:1 or 18:2, 8:2), 34:2(16:0, 18:2 or 16:1,18:1) and 34:1(16;0, 18:1). The prevalence of 18:3 and 16:0 molecular species is not surprisingly, given previous analysis of which showed these to be the most abundant fatty acids in fiber cells.


PC in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
3.000
2.500
2.000
1.500
1.000
0.500
0.000
PC 32:0
PC 34:1
PC 34:2
PC 34:3
PC 34:4
PC 36:1
PC 36:2
PC 36:3
PC 36:4
PC 36:5
PC 36:6
PC 38:2
PC 38:3
PC 38:4
PC 38:5
PC 38:6
PC 40:2
PC 40:3
PC 40:4
PC 40:5
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5


PA in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
3.000
2.500
2.000
1.500
1.000
0.500
0.000
PA 32:0
PA 34:1
PA 34:2
PA 34:3
PA 34:4
PA 34:5
PA 34:6
PA 36:1
PA 36:2
PA 36:3
PA 36:4
PA 36:5
PA 36:6
PA 38:1
PA 38:2
PA 38:3
PA 38:4
PA 38:5
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5


PE in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
3.000
2.500
2.000
1.500
1.000
0.500
0.000
PE 32:0
PE 34:1
PE 34:2
PE 34:3
PE 34:4
PE 36:1
PE 36:2
PE 36:3
PE 36:4
PE 36:5
PE 36:6
PE 38:2
PE 38:3
PE 38:4
PE 38:5
PE 40:2
PE 40:3
PE 42:2
PE 42:3
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5

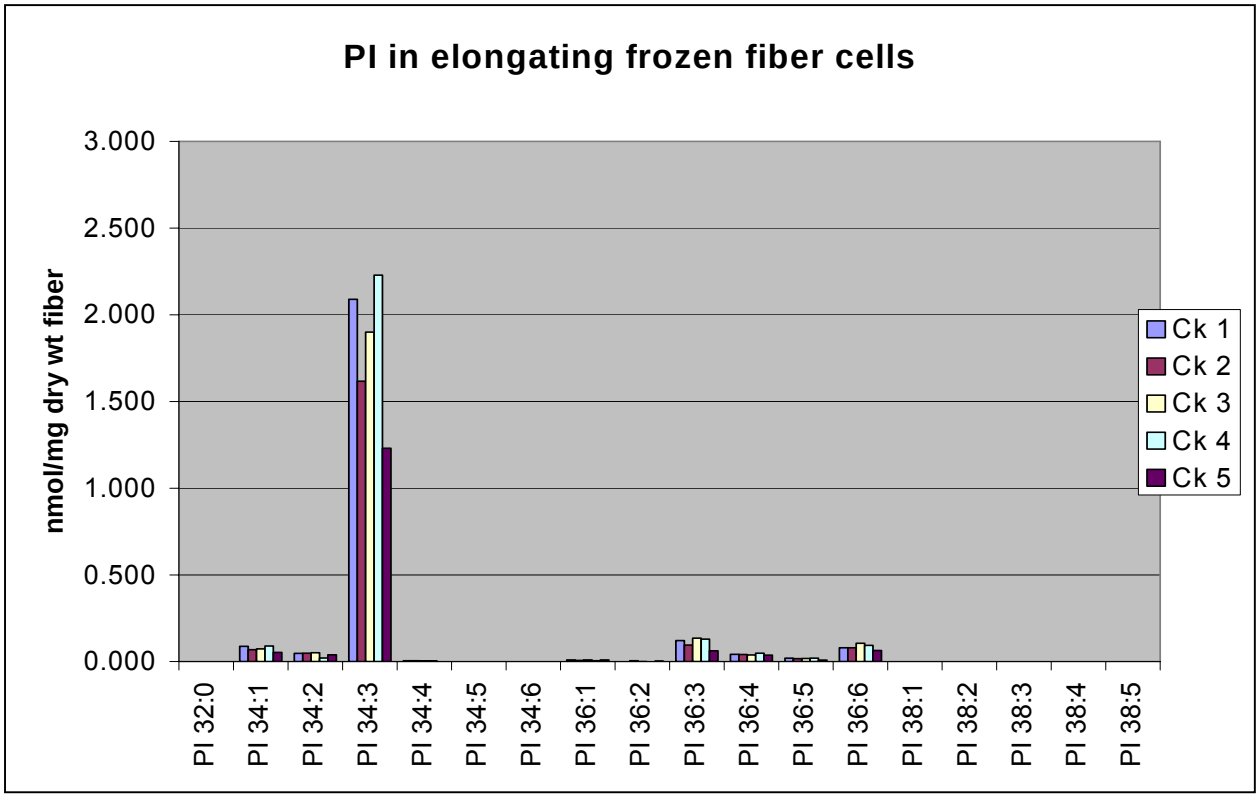
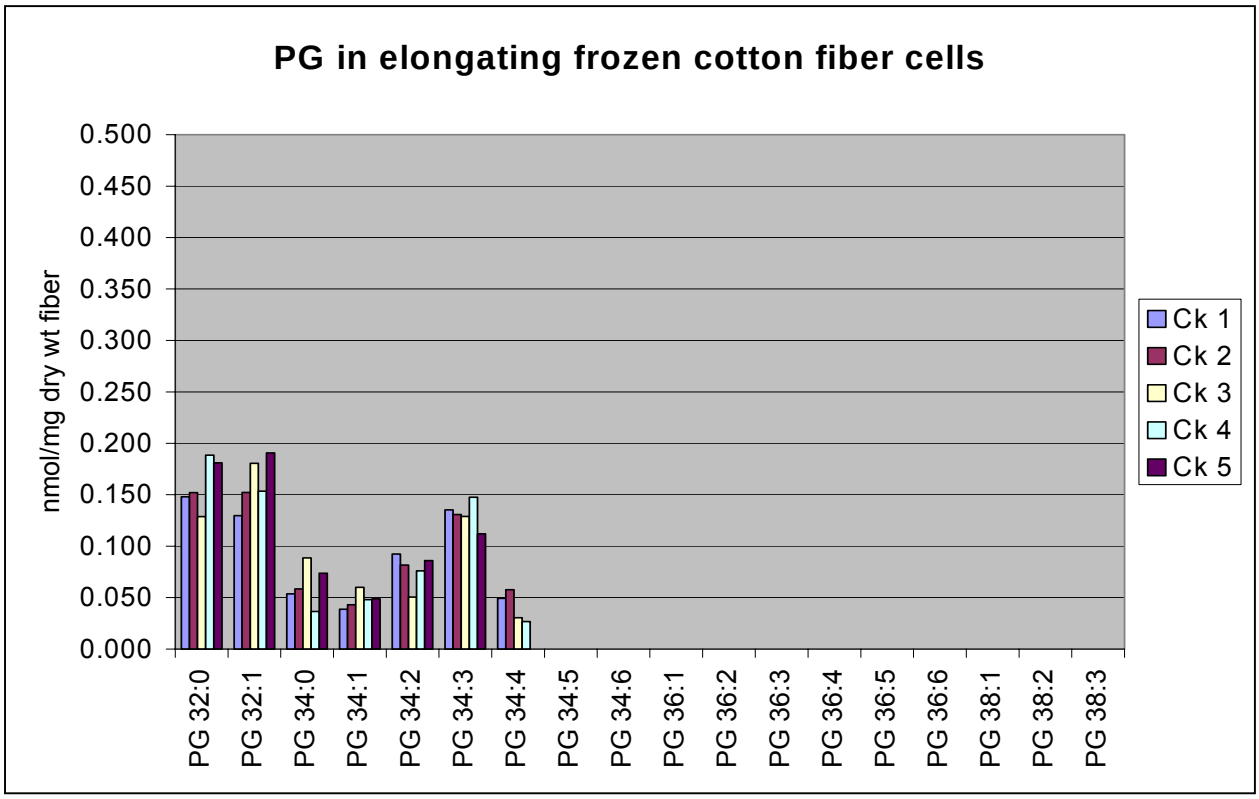
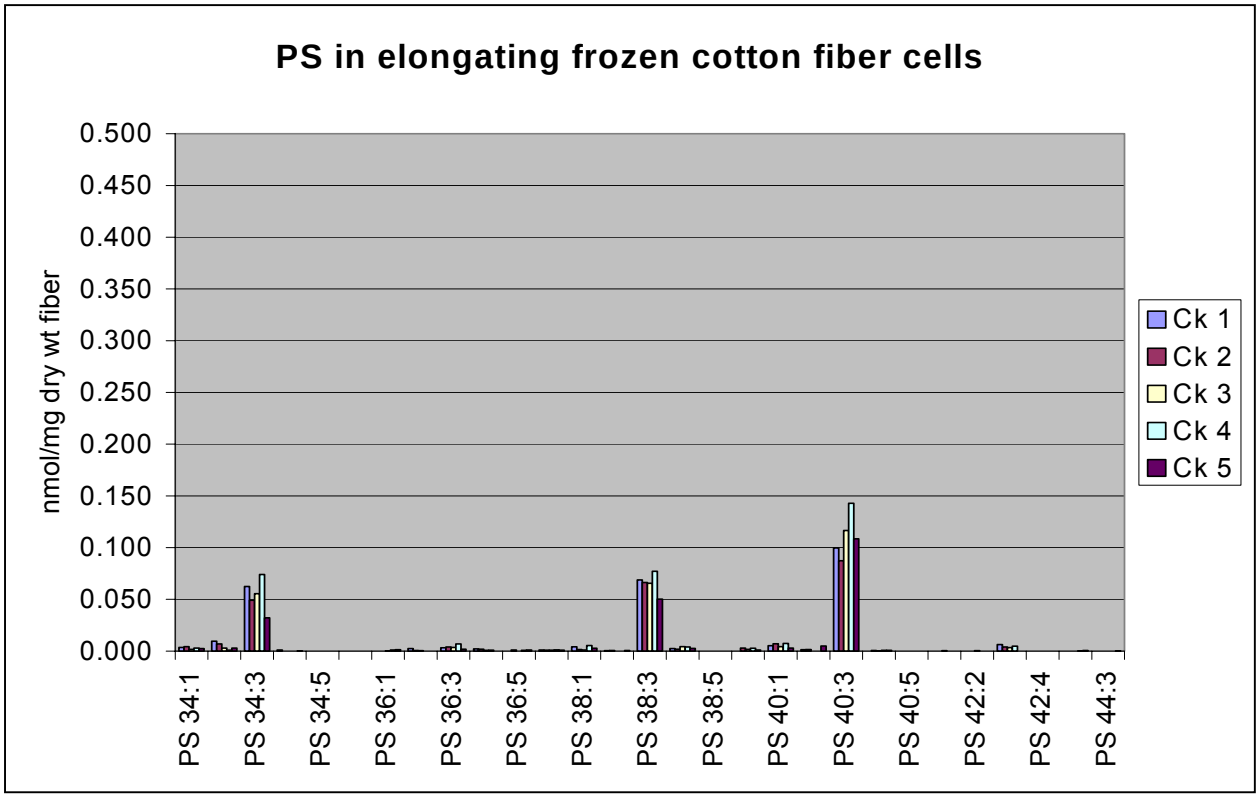

PI in elongating frozen fiber cells
nmol/mg dry wt fiber
3.000
2.500
2.000
1.500
1.000
0.500
0.000
PI 32:0
PI 34:1
PI 34:2
PI 34:3
PI 34:4
PI 34:5
PI 34:6
PI 36:1
PI 36:2
PI 36:3
PI 36:4
PI 36:5
PI 36:6
PI 38:1
PI 38:2
PI 38:3
PI 38:4
PI 38:5
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5

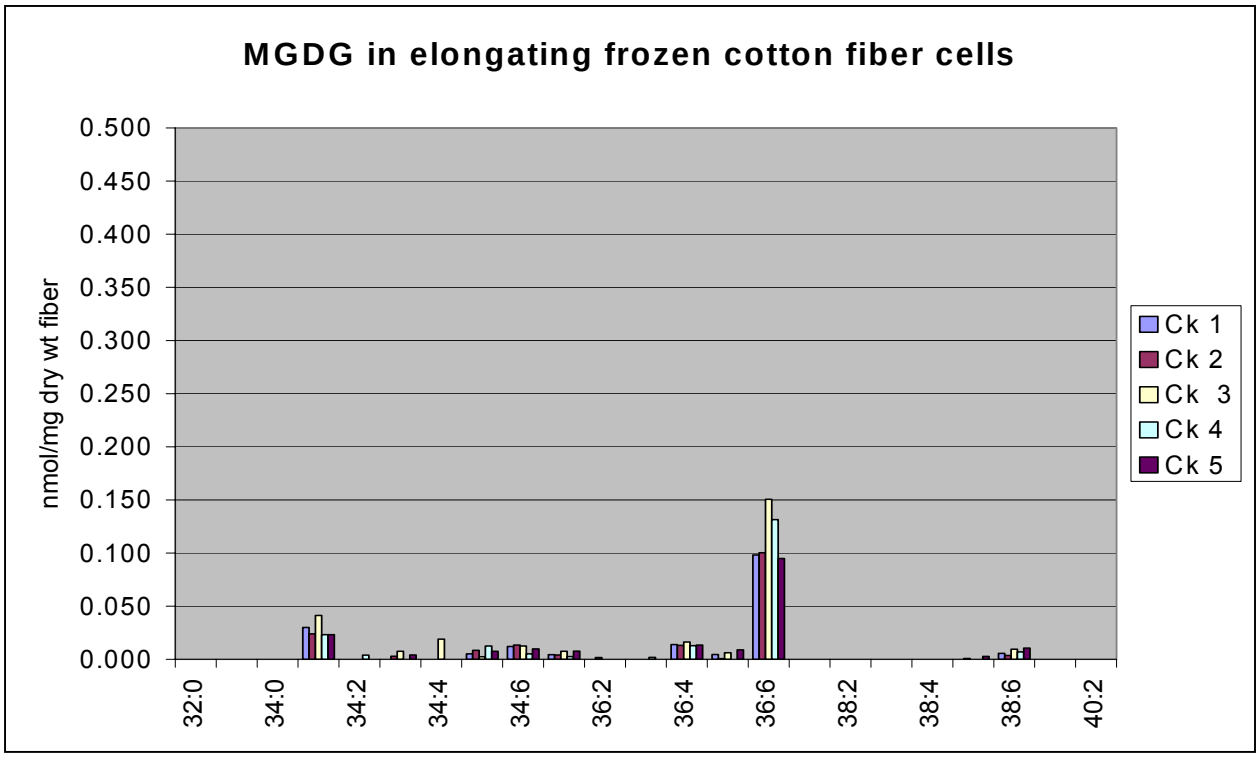
Figure 6: Lipid profiles of elongating cotton (*Gossypium hirsutum* L., cv Coker 312) fiber cells obtained from frozen bolls and analyzed by ESI followed by tandem MS, showing the major membrane glycerolipids. The predominant lipid classes were PC, PE, PI and PA. Similar amounts of PC and PE and PI were observed whereas levels of PA were much higher. The predominant molecular species for each of these phospholipids was 34:3(18:3,16:0) and 36:6(18:3,18:3) was also a fairly abundant species in PC, PE and PA. Other prevalent species present in lower quantities include 36:5(18:3,18:2), 36:4(18:3,18:1 or 18:2, 18:2), 34:2(18:2,16:0 or 18:1,16:1) 34:1(16:0, 18:1). Tissue harvesting and lipid extraction were done at UNT and ESI and tandem MS analysis was performed by Dr Ruth Welti at the Kansas State University Lipidomics Center, Manhattan, Kansas. The bars represent the analysis of 5 individual lipid extracts.

Relatively low levels of PS, PG, MGDG and DGDG, as compared to other membrane glycerolipids, were present in cotton fiber lipid extracts analyzed by ESI and tandem MS (Figure 7). Longer chain fatty acids were found to comprise the major molecular species in PS with PS 40:3 being the most prevalent, while lower levels of PS 38:3 and PS 34:3 were detected. An abundance of PG 32:0(16:0, 16:0), PG 32:1 (16:0,16:1) in elongating fiber as well as PG 34:3(18:3,16:0) was seen in fiber lipid samples. The major molecular species present in MGDG and DGDG was 36:6(18:3, 18:3) while low levels of DGDG 38:6(18:3, 20:3) were observed as well.

The composition of lipids can be used to gain information on how these are synthesized in cells. Glycerolipids are usually synthesized from a PA precursor via two pathways, the prokaryotic and eukaryotic pathway. In the former, 18 carbon fatty acids are esterified to the sn-1 position of the glycerol backbone, while 16 carbon fatty acids are found predominantly in the sn-2 position. In the eukaryotic pathway, 16 carbon fatty acids when present are found at the sn-1 position, while 18 carbon fatty acids can be attached to either position (Frentzen, 1993). PG is produced mainly in plastids, where glycerolipid synthesis occurs via the prokaryotic pathway and this is reflected by the presence of PG 34:3(18:3,16:0). However, other major species of PG 32:0(16:0, 16:0) and 32:1(16:0,16:1) indicate that some PG is produced from PA with a eukaryotic origin, harboring 16:0 at the sn-1. The composition of MGDG and DGDG which both had 36:6(18:3,18:3) as the most abundant species, points to these lipids being synthesized via the eukaryotic pathway, which would be the only mechanism by which 18 carbon chains, are placed at both sn-1 and sn-2 position.


PG in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
PG 32:0
PG 32:1
PG 34:0
PG 34:1
PG 34:2
PG 34:3
PG 34:4
PG 34:5
PG 34:6
PG 36:1
PG 36:2
PG 36:3
PG 36:4
PG 36:5
PG 36:6
PG 38:1
PG 38:2
PG 38:3
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5


PS in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
PS 34:1
PS 34:3
PS 34:5
PS 36:1
PS 36:3
PS 36:5
PS 38:1
PS 38:3
PS 38:5
PS 40:1
PS 40:3
PS 40:5
PS 42:2
PS 42:4
PS 44:3
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5


MGDG in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
32:0
34:0
34:2
34:4
34:6
36:2
36:4
36:6
38:2
38:4
38:6
40:2
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5

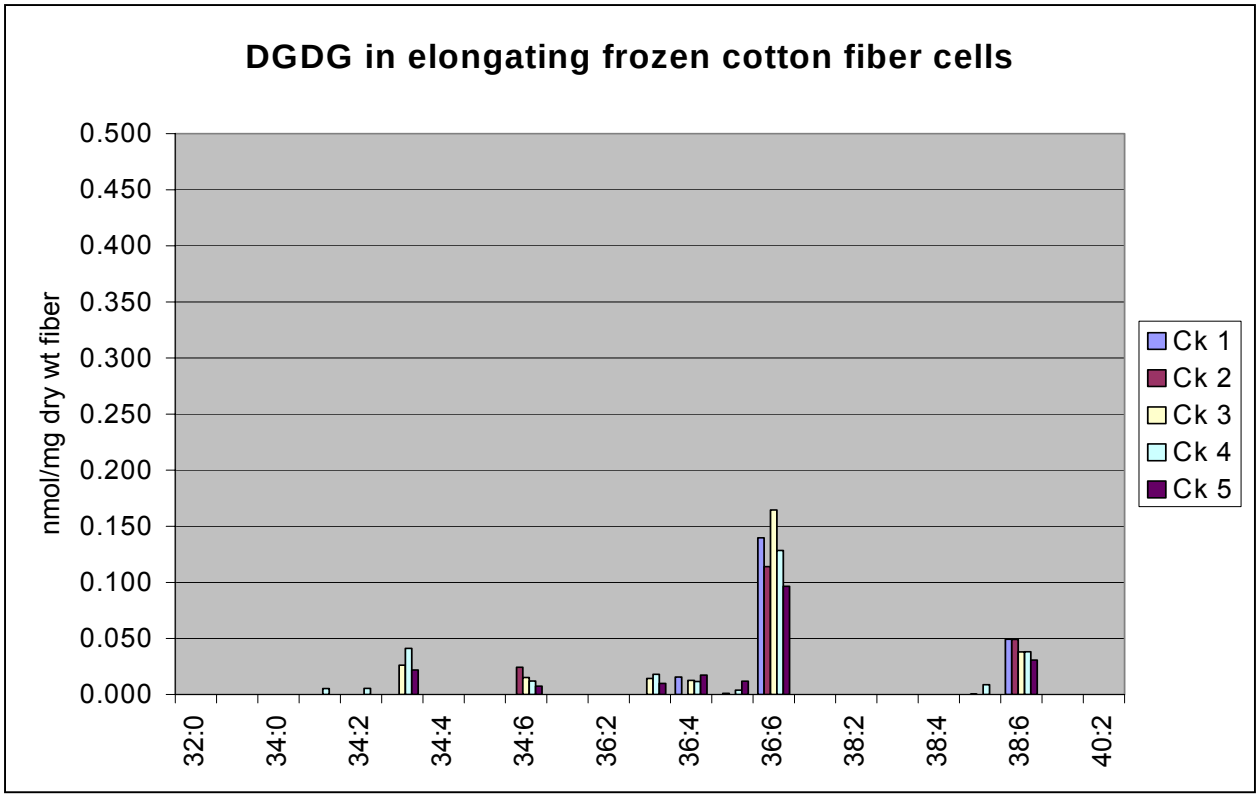

DGDG in elongating frozen cotton fiber cells
nmol/mg dry wt fiber
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
32:0
34:0
34:2
34:4
34:6
36:2
36:4
36:6
38:2
38:4
38:6
40:2
Ck 1
Ck 2
Ck 3
Ck 4
Ck 5

Figure 7: Profiles of minor lipid classes PS, PG, MGDG and DGDG, generated by ESI tandem MS analysis of lipid extracts from frozen cotton fiber (*Gossypium hirsutum,* L. cv Coker 312). PS largely consisted of long chain fatty acids, with a predominance of the molecular species 40:3 (20:3, 20:0) and 38:3(18:3, 20:0), and lower levels of PS 34:3(16:0,18:3). The major molecular species for PG were 32:0(16:0, 16:0), 32:1(16:1,16:0, or 16:0,16:1), and 34:3(18:3,16;0), while less prevalent species 34:0(18;0, 16;0), 34:1(18:1,16:0, or 18:0,16:1), 34:2(18:2,16:0 or 18:0,16:2) and 34:4(18:2,16:2) were also detected. The galactolipids MGDG and DGDG were predominantly composed of 36:6 (18:3,18:3), and other molecular species were barely detectable, with the exception of DGDG 38:6(18:3, 20:3). Tissue harvesting and lipid extraction were done at UNT and ESI and tandem MS analysis was performed by Dr Ruth Welti, at the Kansas State University Lipidomics Center, Manhattan, Kansas. The bars represent analysis of 5 individual lipid extracts.

To determine whether the abundance of PA was a specific feature of fibers, we analyzed lipids in different tissues of the cotton plant. Cotton bolls were harvested, along with their subtending leaves, and immediately placed on dry ice. Lipid extracts were obtained from the fiber, leaf and boll tissue, and analyzed by HPLC. The composition of lipids in leaf was as expected with an abundance of MGDG (58% by weight of total lipids), while DGDG and SQDG comprised12% and 3% by weight of total lipids, respectively. Whereas these galactolipids were absent from lipid extracts of fiber cells, in boll tissue, MGDG and DGDG comprised12% and10% by weight of total lipids, respectively, while the proportion of SQDG was similar to that in leaf tissue. The sterol glycosides ASG and SG were present in relatively low quantities in leaf tissue, comprising less than 1% of total lipids, whereas higher amounts were present in fiber cells and boll tissue. The proportion of ASG and SG in fiber cells was 6% and 4% by weight of total lipids respectively and the levels in boll tissue were similar.

PC was found to be the major membrane phospholipid in leaf tissues, comprising about 13% by weight of the total lipid content, while the other membrane phospholipids. PE and PI made up a much smaller proportion (1% and 6% by weight of total lipids, respectively). In lipid extracts from fiber cells, a relatively high proportion of PI (37% of total lipid weight) was observed, as compared to PC and PE which comprised 9% and 4% by weight of total lipids, respectively. In boll tissues, the quantities of PI were about the same as those in fiber cells, while the level of PC was almost twice as much as that in fiber cells, and the level of PE was about half of that in fiber cells. PA comprised 1% and 12% by weight of lipids present in leaf tissues and boll tissues, respectively. In contrast, in fiber cells an abundance of PA (40% by weight of total lipids) was seen, as

was the case in previous samples. Whether this PA in fiber cells was generated during development or was produced post harvest in fiber was important to reconcile. Nonetheless, either scenario points to cotton fiber as likely to be a rich source of PLD activity.

To determine if the process of freezing cotton bolls on dry ice once harvested, was having an effect on the lipid composition, and producing high levels of PA, fiber samples were obtained from freshly harvested bolls, and lipid extraction was performed immediately, without freezing the tissue. Lipid analysis revealed significantly lower levels of PA as compared to those observed in frozen fibers (Figure 7). These results suggest that PA abundance in frozen fiber samples was generated post harvest due to the freezing process as PA production during lipid extraction has already been ruled out. It is possible that the freezing of fiber cells, which have high moisture content, may result in the formation of ice crystals that destroy subcellular membranes. This may lead to loss of compartmentation of PLD and cause rapid production of PA.

The polar lipid profiles of elongating and maturing fresh cotton fiber cells revealed that PC was the most abundant lipid class, comprising 47% and 36% by weight of total lipids, respectively. PI was also fairly prevalent, and more so in maturing fiber constituting 29%, as compared to 18% by weight of total lipids in elongating fiber. PE and DGDG were present in similar amounts when comparing elongating and maturing fiber, as well as relative to each other. PE in elongating and maturing fiber cells comprised 14% and 13% by weight of total lipids, respectively, while DGDG constituted 12% and 11% by weight of total lipids, respectively. Relatively low levels of PA were observed, with only 2% and 4% by weight of total lipids in elongating and

maturing fiber cells, respectively. The levels of ASG were much higher in maturing cells as compared to that in elongating fiber cells, while similar levels of SG were observed.

The differences in lipid composition observed when comparing lipid extracts from fresh fibers to those from frozen fibers, were common to both elongating and maturing fiber cells (Figure 10). PC was found to be the most abundant phospholipid, comprising 47% and 36% by weight of total lipids, in elongating and maturing fiber cells, respectively, while very low PA levels (less than 5%) were observed in both. This is significantly different from the lipid composition in frozen fibers, especially that observed in maturing fiber cells where PA was the predominant lipid class making up 44% of total lipids while the level of PC was only 15%. In elongating fiber cells the levels of PC and PA also varied considerably when comparing fresh to frozen fibers, constituting 47% and 4% by weight of total lipids respectively, in fresh fiber cells, compared to 26% and 17% by weight of total lipids respectively, in frozen fiber cells. The proportion of PE remained fairly constant in both elongating and maturing fiber cells, regardless of whether the fibers were fresh or frozen. In maturing fiber cells, PI levels observed in fresh fibers were similar to those observed in frozen fibers while in elongating fiber cells much higher PI levels were observed in frozen fiber cells which comprised 39% as compared to 18% by weight of total lipids in fresh fiber cells. The levels of DGDG about 12% of total lipid weight in fresh fiber cells were, about 6 fold higher than those observed frozen fiber cells in both elongating and maturing cells.

Freezing of fiber cells severely altered the levels of PC and we hypothesized that this was probably due PLD mediated hydrolysis producing the high levels of PA observed in these cells. Cotton fiber cells from maturing bolls were used to obtain crude

enzyme extracts. PLD activity in these crude extracts was measured as hydrolysis of radioactive PC (L-α-phosphatidylcholine dipalmitoyl, [dipalmitoyl-1-^{14} C]) to PA and transphosphatidylation to phosphotidylethanol (PtdEtOH). PLD activity measured under conditions optimal for PLD α was found to be 1.48μmol/min/mg protein, which is very high, compared to reported pld activity in other plant cells. Freezing of fibers post harvest may result in high PLD activity and may explain why such a high amount of PA was found in lipid extracts from frozen fibers.

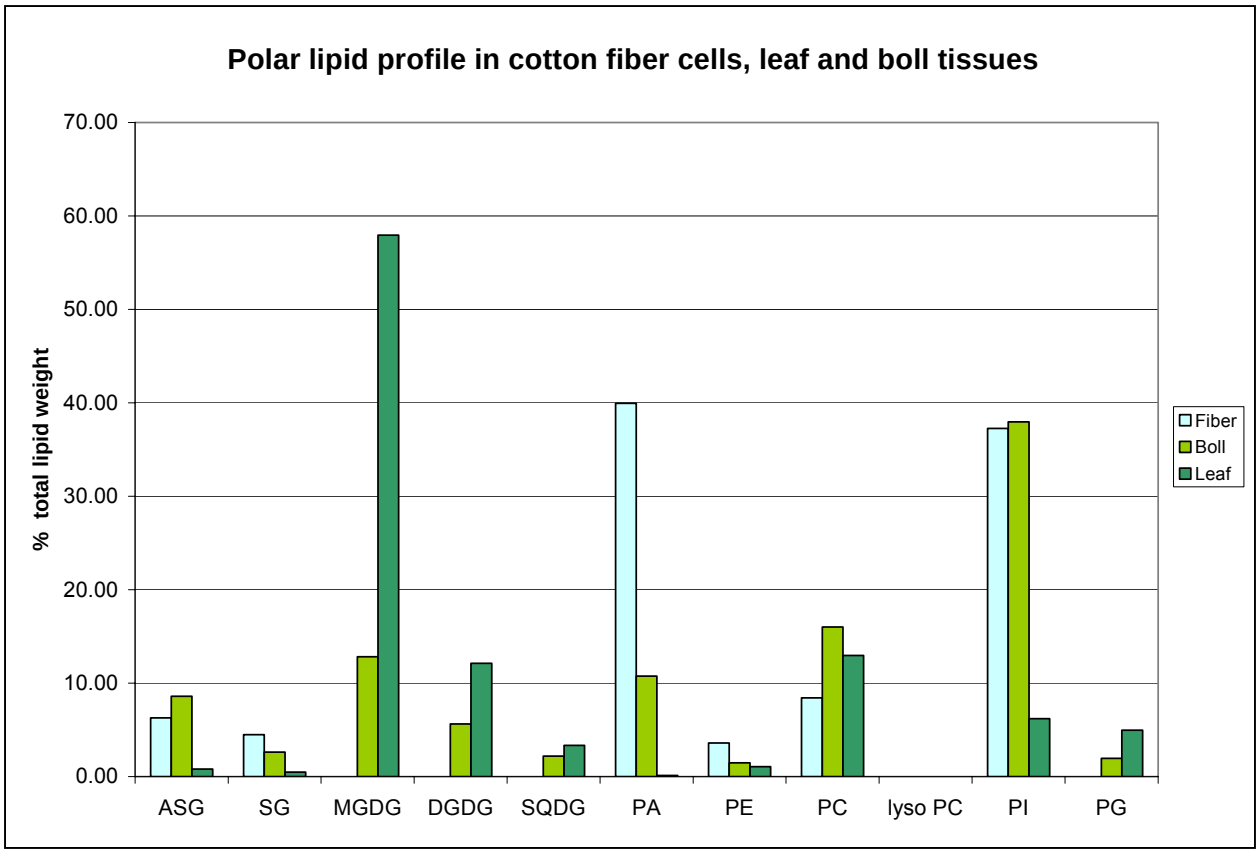


Figure 8: Profile of polar lipids in different cotton tissues: elongating frozen cotton fiber, boll tissues and leaf tissues, analyzed by HPLC using ELSD and MS. The lipid composition of leaf tissues showed an abundance of galactolipids, with MGDG being the most prevalent. PC was found to be major phospholipid in leaf tissues, while lower levels of PI and PE were detected. Higher amounts of PI as compared to PC and PE were present in lipid extracts from fibers, and a similar pattern was observed in boll tissues. Very low levels of PA were found in leaf tissues as compared to fiber cells. The levels of most lipid classes in boll tissues, was intermediate to the levels in fiber cells and leaf tissues. Dr Robert A. Moreau, USDA-ARS-Eastern Regional Research Center, Philadelphia, PA performed the HPLC analysis of tissue lipid extracts prepared at UNT. Values represent the results from a single experiment.

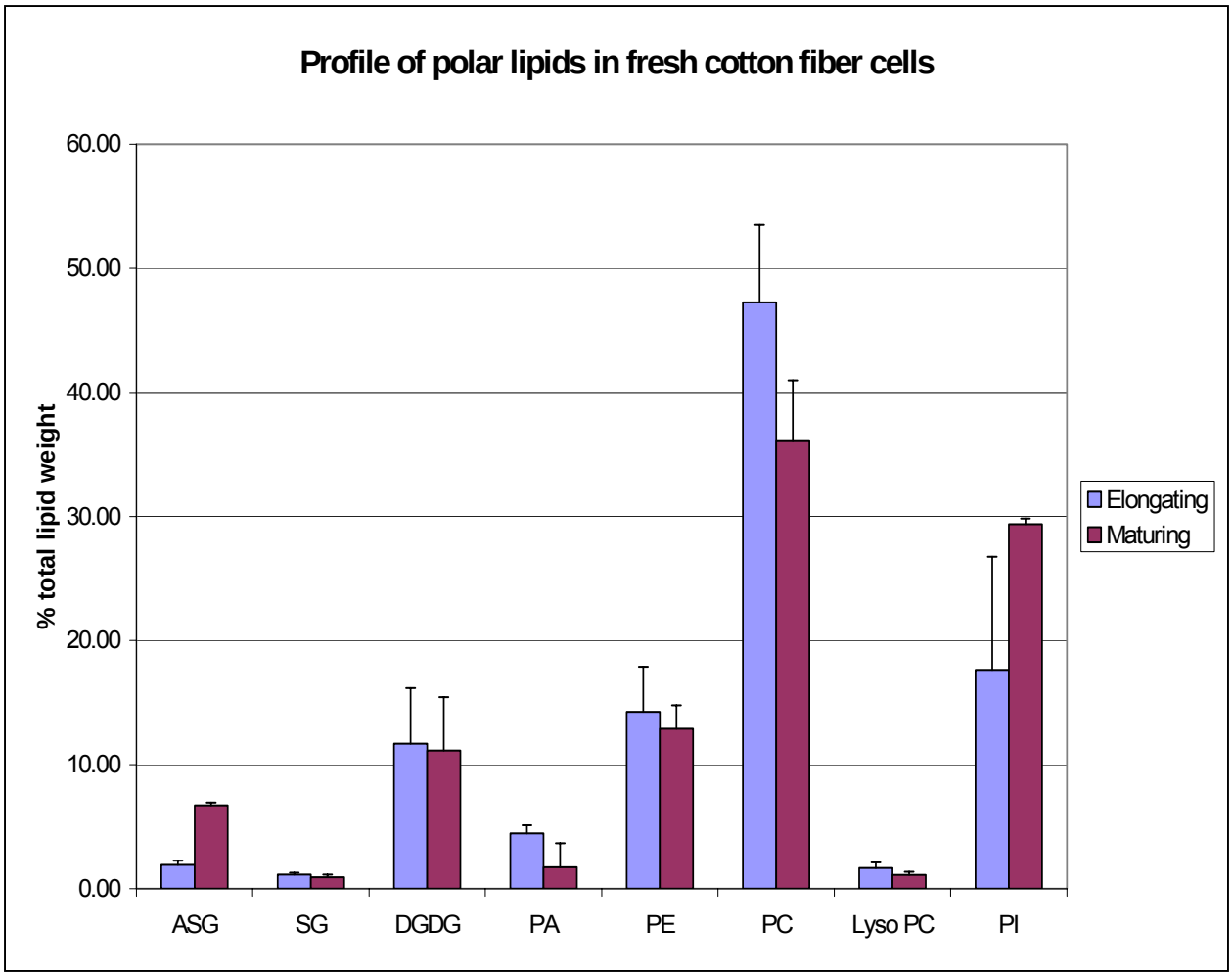
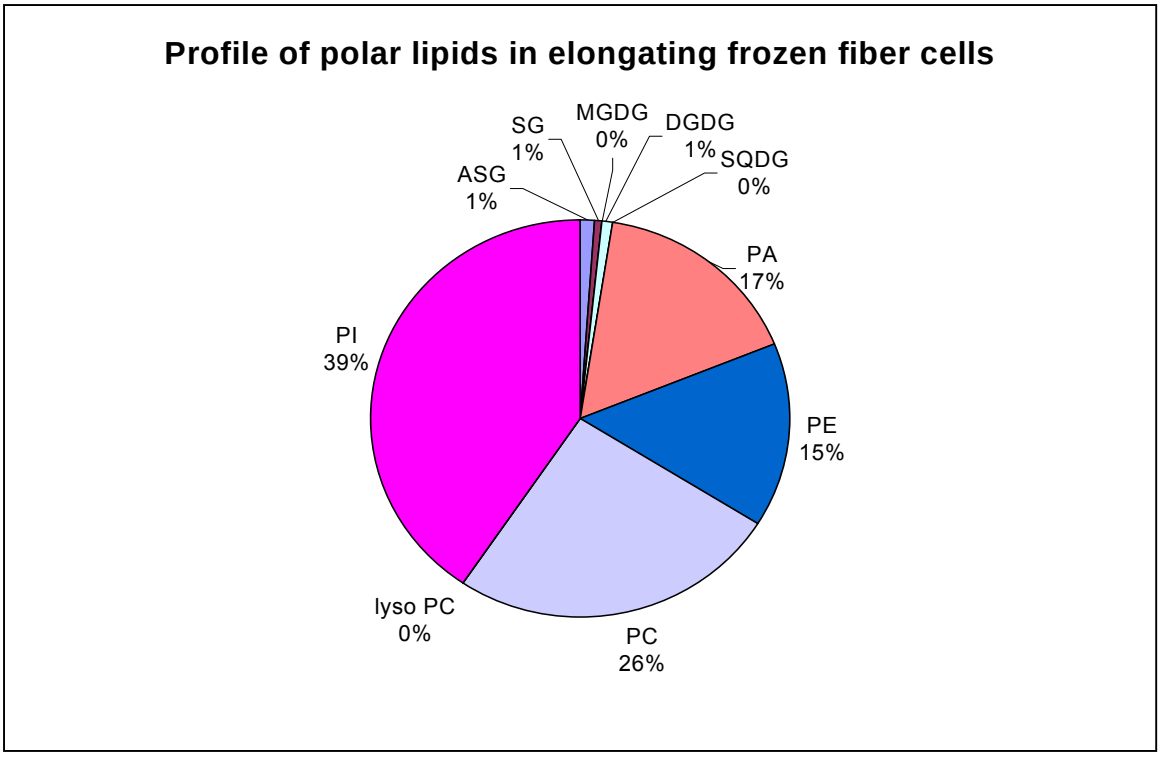
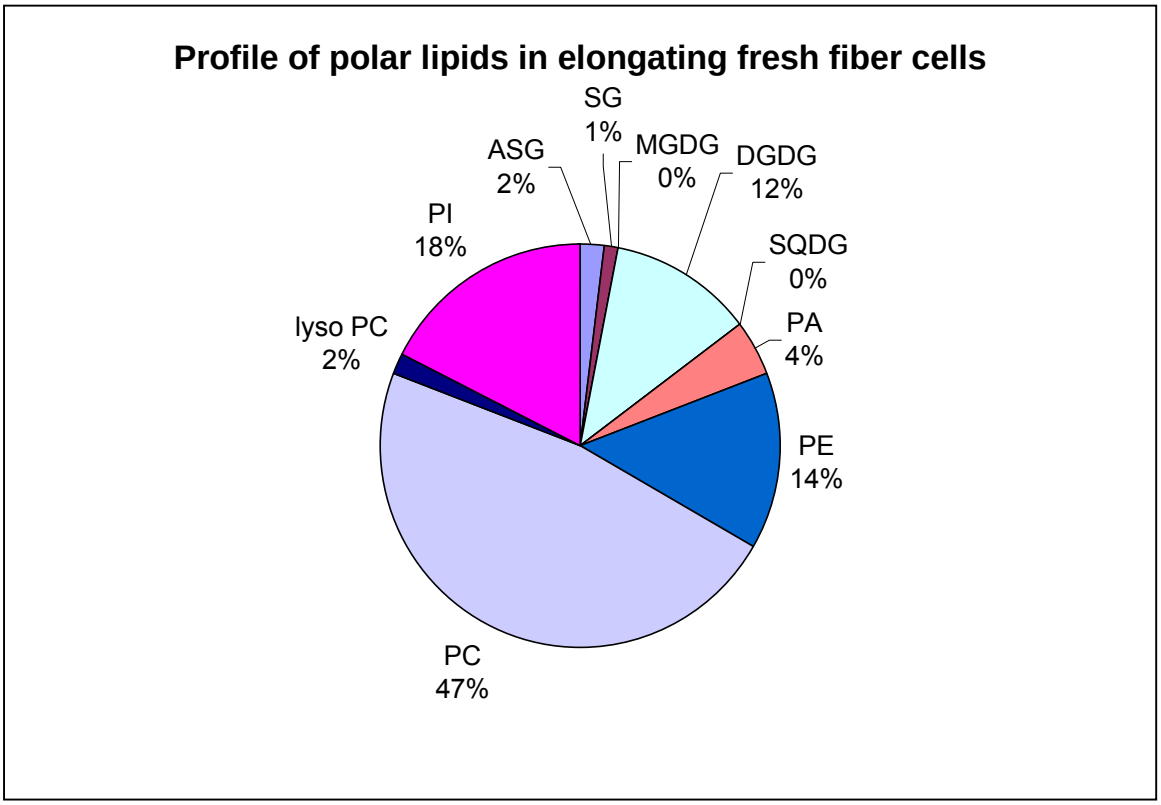

Profile of polar lipids in fresh cotton fiber cells
% total lipid weight
60.00
50.00
40.00
30.00
20.00
10.00
0.00
ASG
SG
DGDG
PA
PE
PC
Lyso PC
PI
Elongating
Maturing

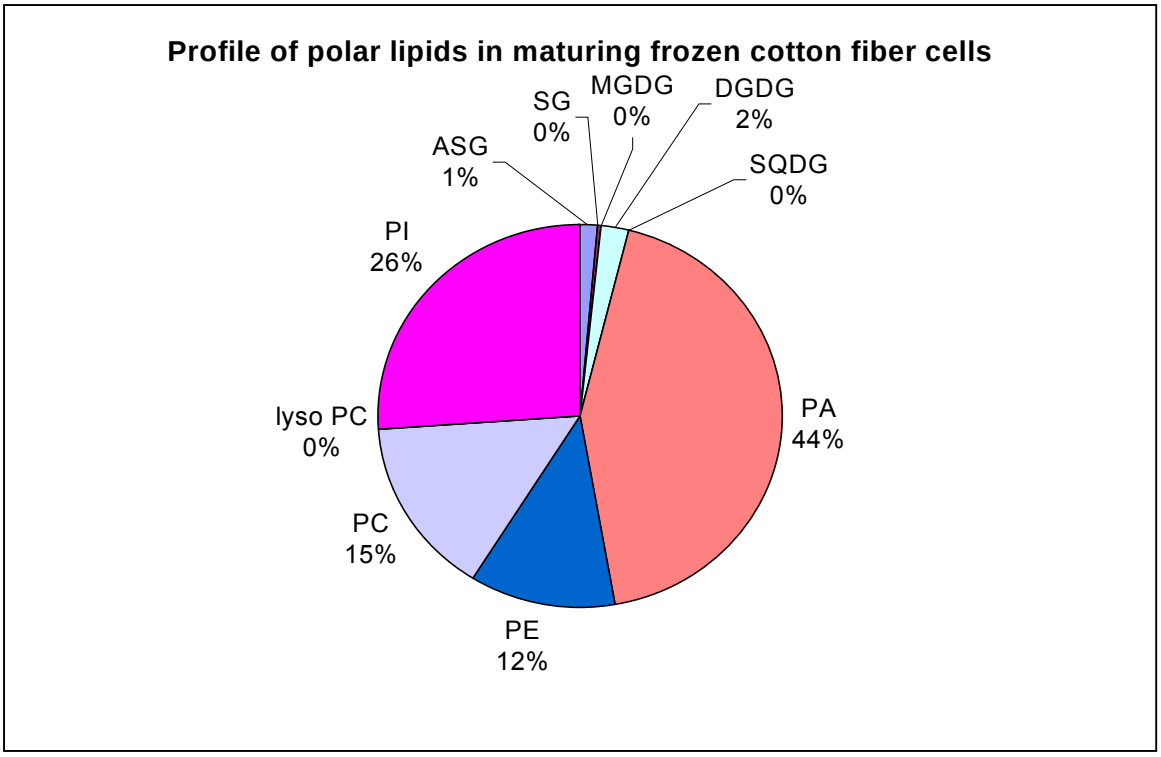
Figure 9: Comparison of polar lipid profiles of elongating fresh and maturing fresh fiber. PC was found to be the most abundant lipid class, with a larger proportion found in elongating fiber (47%) as compared to that in maturing fiber (36%). PI was also fairly prevalent with a higher proportion (29%) found in maturing fiber as compared to that in elongating fiber (18%). The levels of PE were relatively similar in elongating and maturing fiber comprising 14% and 13% by weight of total lipids respectively, as were the levels of DGDG, which made up 12% and 11% by weight of total lipids, respectively. PA levels were fairly low in both elongating and maturing fiber, constituting less than 5% by weight of total lipids. The levels of ASG were higher in maturing fiber 7% as compared to 2% of total lipid weight in elongating fiber, while similar levels of SG were observed. A small amount of lysophosphatidylcholine (lysoPC) was observed in both elongating and maturing fiber. Bars represent the means and SD of three to four independent extractions. Dr Robert A. Moreau, USDA-ARS-Eastern Regional Research Center, Philadelphia, PA performed the HPLC analysis of tissue lipid extracts prepared at UNT.

A.


Profile of polar lipids in elongating frozen fiber cells
SG 1%
MGDG 0%
DGDG 1%
SQDG 0%
ASG 1%
PA 17%
PI 39%
PE 15%
lyso PC 0%
PC 26%


Profile of polar lipids in elongating fresh fiber cells
SG 1%
ASG 2%
MGDG 0%
DGDG 12%
PI 18%
SQDG 0%
lyso PC 2%
PA 4%
PE 14%
PC 47%

B.


Profile of polar lipids in maturing frozen cotton fiber cells
SG 0%
MGDG 0%
DGDG 2%
ASG 1%
SQDG 0%
PI 26%
PA 44%
lyso PC 0%
PC 15%
PE 12%

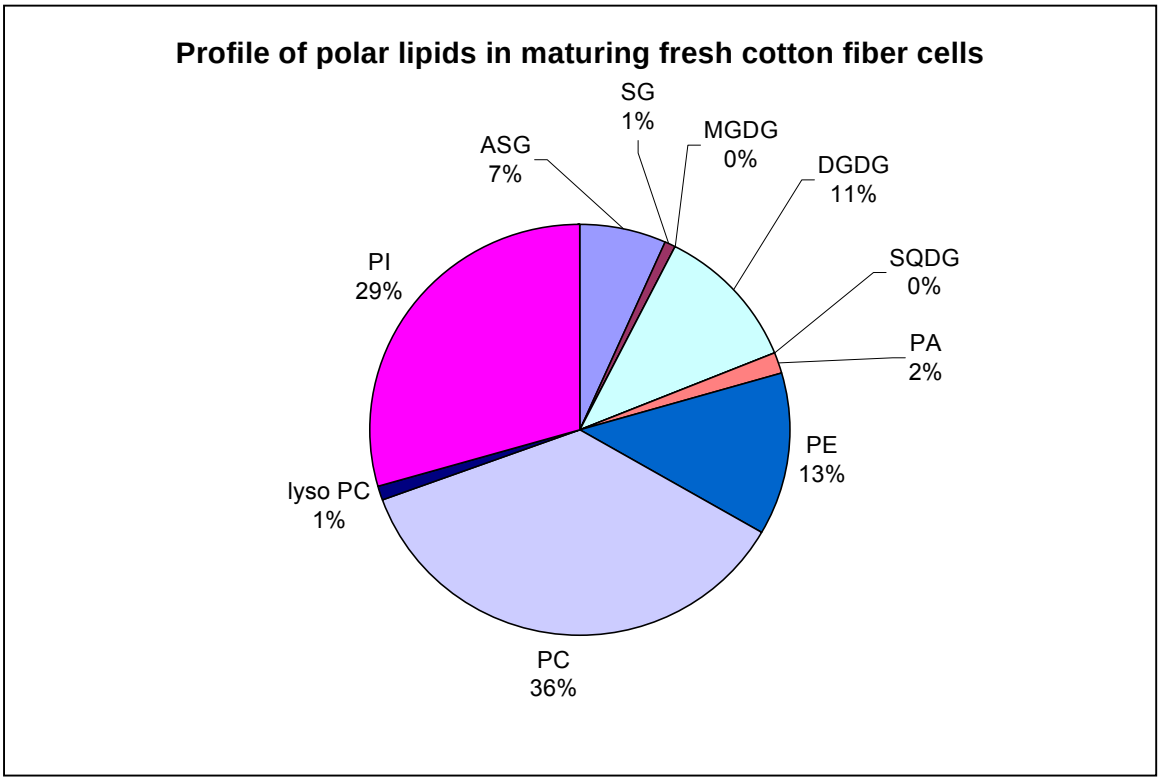

Profile of polar lipids in maturing fresh cotton fiber cells
SG 1%
MGDG 0%
ASG 7%
DGDG 11%
PI 29%
SQDG 0%
PA 2%
PE 13%
lyso PC 1%
PC 36%

Figure 10: Comparison of the lipid profiles of frozen fibers (Figure 4) to those of fresh fibers (Figure 9) for elongating and maturing fiber cells. The most significant difference is seen in the relative amounts of PC and PA. Whereas relatively higher proportions of PA are seen in frozen fiber cells, an abundance of the PC was observed in fresh tissue, in which PA comprised less than 5% by weight of total lipids. Similar levels of PE were noted in both fresh and frozen tissue. PI levels varied when comparing elongating to maturing fiber cells. A. In maturing fiber cells, PI levels were about the same in fresh fibers as compared to frozen fibers. B. In elongating fiber cells higher PI levels were observed in frozen fibers as compared to fresh fibers. DGDG comprised a larger proportion in fresh fibers than frozen fibers, as did ASG and SG.

Profile of Expressed Sequence Tags in Developing Cotton Fibers

Several genes in cotton have been isolated and sequenced, and many of these genes have been shown to be preferentially expressed in cotton fiber, in addition to having a temporal pattern of expression that correlates well with the different developmental stages (John and Crow, 1992, Song and Allen, 1996, Ma et al., 1995, Orford and Timmis, 1997, 1998 and 1999, Zhao et al., 2001, Li et al., 2002, Ji et al., 2003). Gene expression in cotton has also been examined and expressed sequence tags (ESTs) from different tissues in cotton have been assembled at The Institute for Genomic Research Website at http://www.tigr.org. These include ESTs from two libraries for cotton fibers at 7 to10dpa and 6dpa, which is the stage of rapid elongation. We used this data to catalog ESTs for the major enzymes involved in lipid metabolism in elongating cotton fiber (Table 1). To date 38814 ESTs have been obtained from the 7 to 10dpa library, as compared to only 8003 from the 6dpa library. Hence, the number of ESTs and tentative consensus sequences (TCs) discussed here are almost exclusively those of the larger (7 to10dpa) library, with certain noted exceptions. In some cases the ESTs were already annotated, while others were identified using a BLAST program from the TIGR Website with cDNA and protein sequences from other plants as queries. Many of these plant sequences were obtained from a lipid gene database housed at http://www.plantbiology.msu.edu/lipids/genesurvey/index.htm, for *Arabidopsis thaliana* (Beisson et al., 2003), hence, we also compared the total number of lipid enzyme ESTs from all tissues in *A. thaliana*, to those in cotton fiber cells. The abundance of ESTs was used only as an indicator of the potential of fiber cells to produce the different lipids enzymes, which in turn produce the respective lipid metabolites.

Table 1: Catalog of expressed sequence tags (ESTs) predicted to encode for enzymes and other proteins involved in lipid metabolism in two cotton fiber libraries (7-10dpa and 6dpa). The number of tentative consensus sequences (TCs) in the 7-10dpa library is noted as an indicator of the potential number of genes for each protein. The EST data was obtained from Institute for Genomic Research (TIGR) through the Website at http://www.tigr.org. The EST data from the TIGR Cotton Gene Index was accomplished with support from the National Science Foundation. Comparisons were made to ESTs involved in lipid metabolism in *A. thaliana*, using data compiled in the Arabidopsis lipid gene database housed at www.plantbiology.msu.edu/lipids/genesurvey/index.htm (Beisson et al., 2003). Lipid metabolic genes for which no ESTs have been annotated in this database are marked as N/A.

	Enzyme or protein	**Cotton fiber**			**Arabidopsis (all tissues)**	
		ESTs	**ESTs**	**TCs**	**ESTs**	**Genes**
EC #	**Fatty Acid Biosynthesis**	**7-10 dpa**	**6dpa**	**7-10 dpa**		
6.4.1.2	ACCase	47	8	**6**	68	**1**
	Fiber specific ACP	7	2	**1**		
	ACP plastidial isoform 1	12	0	**1**	42	**5**
	ACP plastidial isoform 2	5	1	**1**		
	ACP mitochondrial isoform	9	1	**3**	12	**3**
2.3.1.39	ACP S-malonyltransferase	9	0	**1**	3	**3**
2.3.1.41	Ketoacyl-ACP synthase 1	40	18	**5**	39	**1**
2.3.1.41	Ketoacyl-ACP synthase II	0	0	**0**	2	**1**
2.3.1.41	Ketoacyl-ACP synthase III	0	0	**0**	6	**1**
1.1.1.100	B-ketoacyl-ACP reductase	3	2	**1**	35	**5**
4.2.1.*	B-hydroxyl-ACP dehydratase	2	3	**1**	7	**2**
1.3.1.9	Enoyl-ACP reductase	5	2	**2**	11	**1**
1.14.19.2	Stearoyl-ACP desaturase	22	4	**5**	43	**7**
3.1.2.14	Palmitoyl-ACP thioest (FATB)	11	1	**3**	35	**1**
3.1.2.14	Oleoyl-ACP hydrolase (FATA)	3	0	**1**	5	**2**

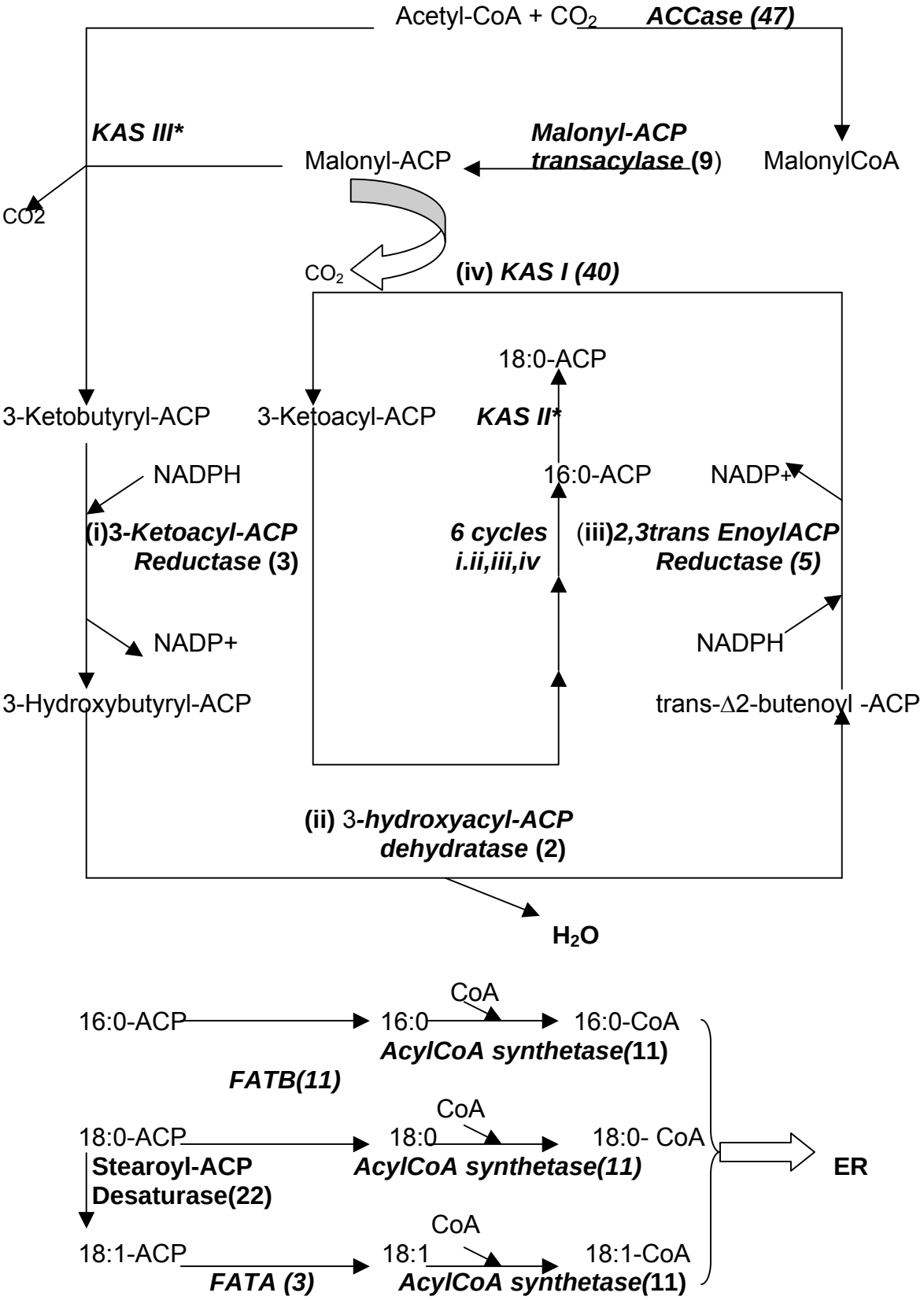
	Glycerolipid Synthesis					
	Acyltransferase	66	17	**3**		
2.3.1.15	Acyl-ACP G3P acyltransferase	1	0	**0**	2	**1**
2.3.1.15	Acyl-CoA G3P acyltransferase	9	0	**1**	2	**2**
2.3.1.51	LPAAT-plastidial	13	0	**1**	7	**1**
2.3.1.51	LPAAT-ER (Acyl-CoA:1acylG3PAT)	7	3	**1**	5	**11**
2.7.7.41	Phosphatidic acid phosphatase	1	2	**0**	2	**1**
2.7.1.107	DAG kinase-like protein	4	1	**2**	33	**8**
2.3.1.20	DAGAT	0	0	**0**	7	**2**
2.7.7.41	CDP: DAG synthetase	0	0	**0**	4	**3**
2.7.8.5	PGP synthase(CDP: DAG G3P phosphtransferase)	2	0	**1**	3	**1**
2.7.8.5	ER PGP synthase	0	1	**0**	7	**1**
3.1.3.27	PGP phosphatase	0	0	**0**	0	**0**
2.7.8.11	PI synthase	0	0	**0**	6	**2**
2.7.1.32	Choline/Ethanolamine kinase	0	3	**0**	13	**4**
2.7.7.15	Phospholipid cytidyltransferase	4	0	**1**	8	**2**
2.7.8.2	Aminoalcoholphosphotransferase	2	0	**0**	11	**1**
1.14.99.*	Omega-6 fad ER (FAD2)	10	10	**2**	131	**1**
1.14.99.*	Plastidial FAD 6	1	0	**0**	9	**1**
1.14.99.*	Omega-3-fad ER (FAD3)	5	4	**1**	31	**4**
1.14.99.*	Plastidial (FAD7/FAD8)	8	1	**1**	18	**2**
2.7.1.46	MGDG synthase	0	0	**0**	5	**3**
2.4.1.184	DGDG synthase	2	0	**0**	8	**2**
	Sphingolipid Biosynthesis					
2.3.1.50	Serine palmitoyltransferase	4	2	**2**	13	**3**
1.1.1.102	3-Ketosphinganine reductase	29	2	**0**	18	**2**
	Acyl-CoA indp ceramide synthase	1	1	**0**	0	**0**
2.3.1.24	Sphinganine acyltransferase	0	0	**0**	0	**0**
	Delta 8 sphingolipid desaturase	1	0	**1**	36	**2**
	Fatty acid elongation/					
	Wax synthesis					
6.2.1.3	Long chain acyl-CoA synthetase	11	4	**2**	63	**9**
	B-ketoacylCoA synthase	150	43	**16**	183	**20**
	B-ketoacyl reductase	15	7	**2**	47	**2**
4.2.1.17	3-hydroxylCoA dehydrase	0	0	**0**	0	**0**
1.3.1.44	Enoyl-CoA reductase	0	0	**0**	31	**1**
	CER1-aldehyde decarboxylase	24	5	**1**	36	**5**
	CER2	0	1	**0**	28	**3**

	CER3	0	5	**0**	7	**1**
	Lipid transfer protein precursor	151	254	**4**	362	**14**
	Lipid transfer protein3 precursor	67	125	**4**	28	**8**
	Palmitoyl-protein thioes like protein	2	2	**1**	44	**7**
	Sterol Biosynthesis					
1.1.1.34	HMG-CoA reductase	21	0	**3**	N/A	N/A
	24-sterol C-methyltransferase	14	11	**1**	N/A	N/A
	Sterol C14 reductase FACKEL	2	0	**1**	N/A	N/A
	Delta 8 delta 7 isomerase	2	0	**1**	N/A	N/A
	Sterol-C4-alpha methyl oxidase	5	2	**1**	N/A	N/A
	UDP:glucose sterol glucosyl transferase	6	2	**2**	N/A	N/A
	SAM Sterol C-methyltransferase	8	0	**1**	N/A	N/A
	Lipases					
3.1.4.4	Phospholipase D (alpha)	25	3	**1**	35	**4**
3.1.4.4	Phospholipase D (Beta1)	2	0	**1**	4	**2**
3.1.4.4	Phospholipase D (Gamma)	1	0	**0**	13	**3**
3.1.1.32	Putative phospholipase A1	2	0	**1**	2	**1**
3.1.1.4	Putative phospholipase A2	7	0	**1**	6	**2**
3.1.1.5	Lysophospholipase homolog	84	11	**18**	28	**9**
3.1.4.11	Phospholipase C	1	3	**0**	11	**6**
3.1.4.11	PI-specific Phospholipase C	12	1	**3**	24	**9**
	Fatty acid amide hydrolase	16	7	**5**	5	**2**
	B-oxidation					
1.3.3.6	Acyl-Co A oxidase	4	1	**2**	61	**6**
4.2.1.17	EnoylCoA hydratase	7	1	**1**	6	**2**
1.1.1.35	3-hydroxybutyrl-CoA dehydrogenase	3	0	**1**	10	**1**
2.3.1.16	3-ketoacylCoA thiolase	5	0	**1**	65	**3**
	AcetyCoA C-acyltransferase	8	0	**2**	0	**0**

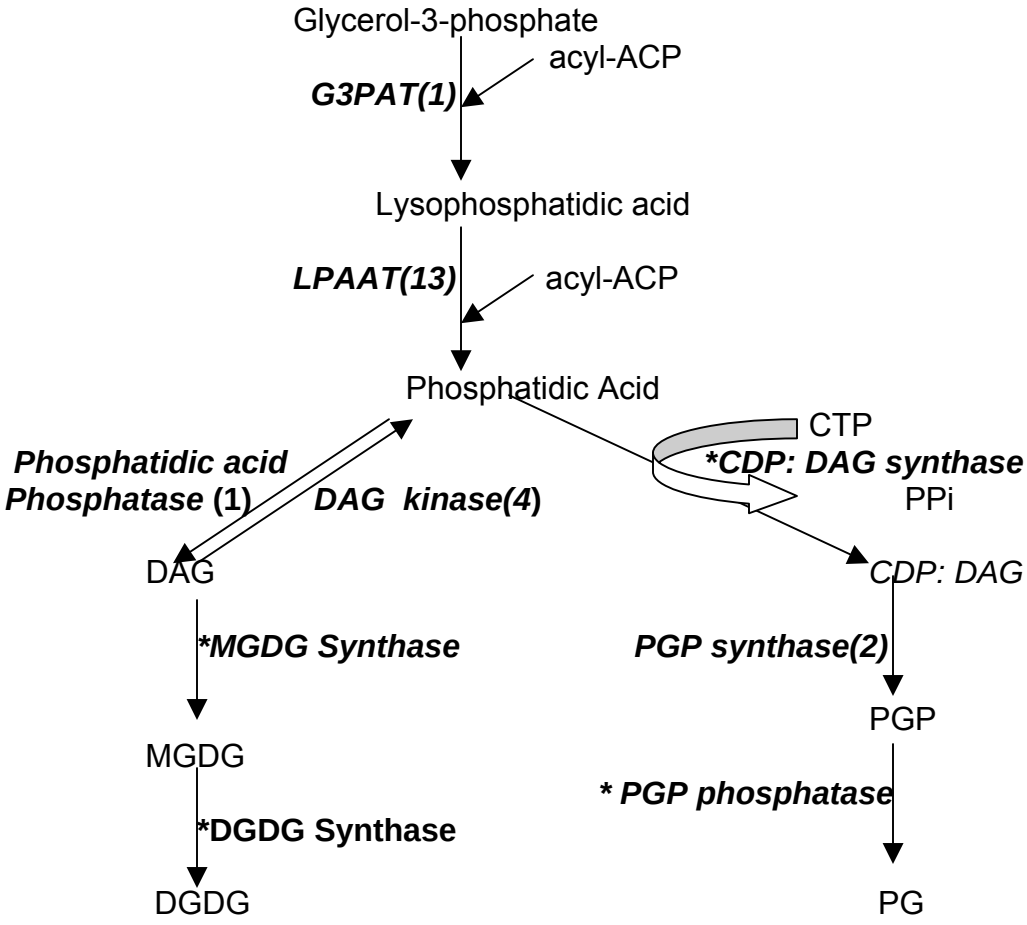
Expression of genes for most of the enzymes involved in fatty acid biosynthesis and acyl chain termination was evident by the presence of several ESTs for these enzymes (Figure 11 A). The most abundant ESTs identified were those predicted to encode for Acetyl-Co A carboxylase (ACCase), acyl-carrier protein (ACP), β-ketoacyl-CoA synthase I (KAS I), and Stearoyl-ACP desaturase. Several ESTs were predicted to encode for different isoforms of ACP, included a fiber specific ACP (Song and Allen, 1997). No ESTs were annotated as encoding for KAS II and KAS III, but these two proteins share high homology with KAS I, and some of the ESTs predicted to encode for KAS I could infact encode for the other two enzymes. These results in addition to metabolic data confirm that cotton fiber cells at this stage of development are actively involved in fatty acid biosynthesis.

Fatty acids that are synthesized in the plastids can either be transferred from acyl-ACPs into plastidial glycerolipids, or cleaved by a thioestrerase to form free acyl chains (Somerville et al., 2000). Three ESTs for FatA, the thioesterase that acts on unsaturated fatty acids, and 11 ESTs for Fat B, which mainly cleaves saturated acyl-ACPs, were identified. Eleven ESTs were annotated as encoding for acylCoA synthetase, which esterifies the free acyl chains to CoA forming acyl-CoAs (Somerville et al., 2000). Glycerolipid synthesis begins with transfer of an acyl chains onto glycerol 3-phosphate (G3P) forming lysophosphatidic acid (LPA), in a reaction is catalyzed by a G3P acyltransferase (Somerville et al., 2000). Several ESTs, were annotated as encoding for acyltransferases. The sequence homology of these ESTs, to that of known plant acyltransferases was queried using the TIGR BLAST program. Only a single EST from the 6dpa library, could be annotated as encoding an acyl-ACP acyltransferase.

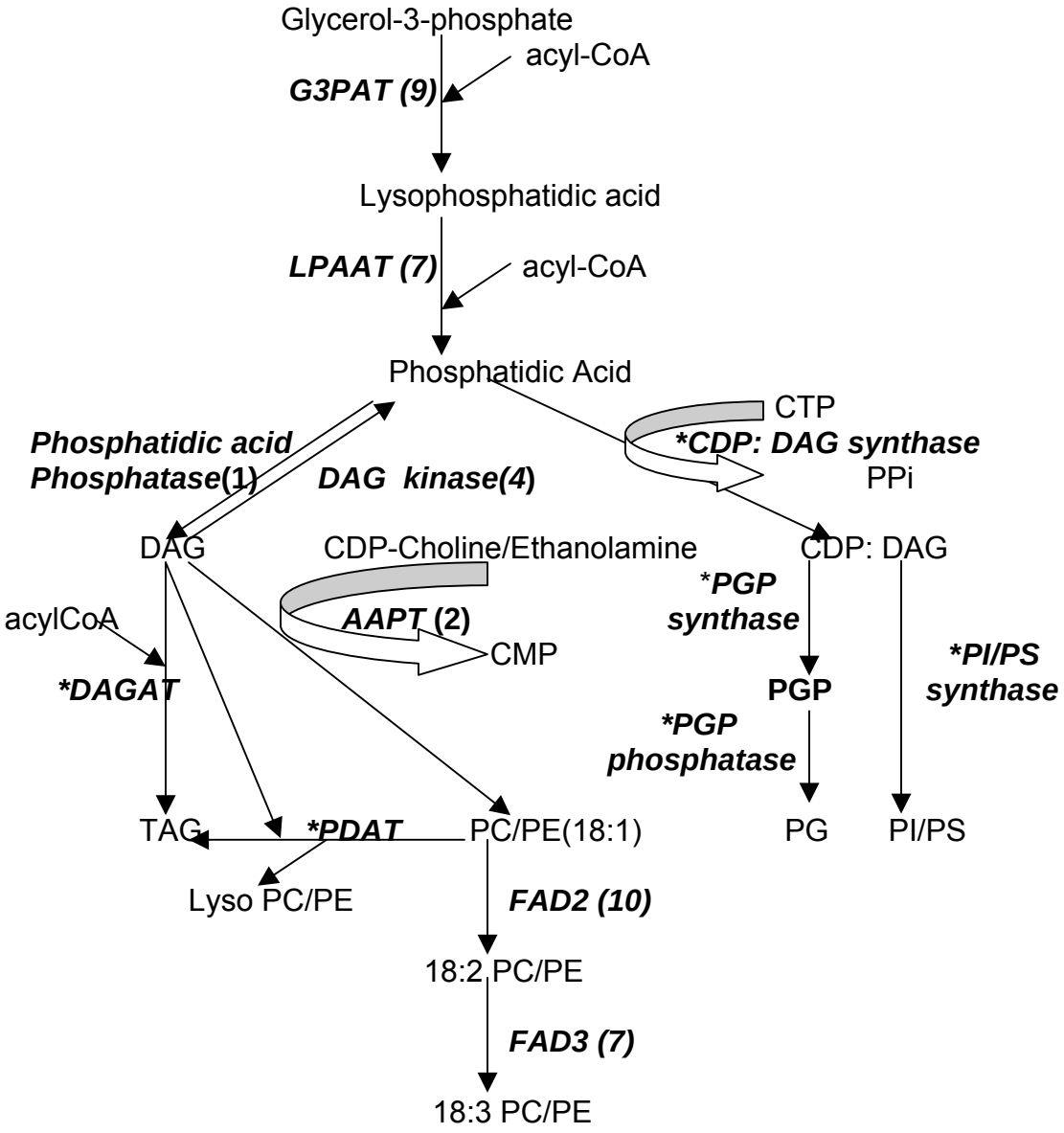
A. **FATTY ACID BIOSYNTHESIS PATHWAY**



B. **GLYCEROLIPID SYNTHESIS (Plastidial)**



C. **GLYCEROLIPID SYNTHESIS (ER)**



D. PHOSPHOLIPID SYNTHESIS VIA CDP: DAG PATHWAY IN THE ER

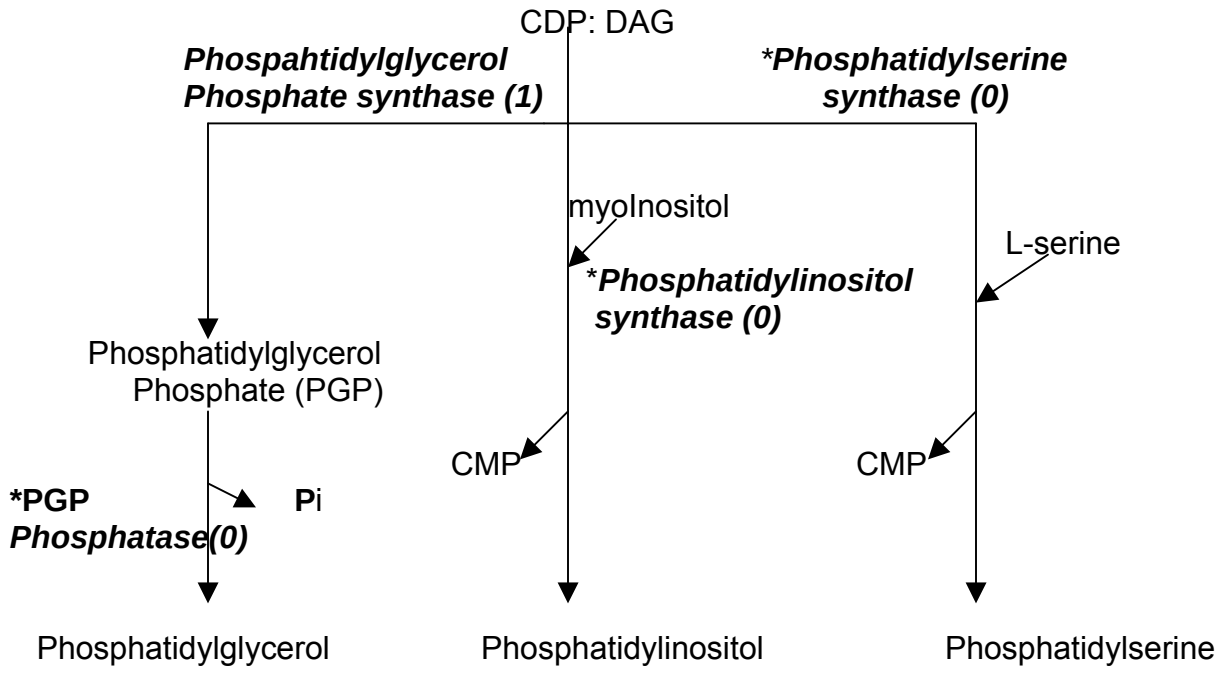


Figure 11: Predicted metabolic schemes for synthesis of fatty acids and glycerolipids in cotton fiber cells developed based on the data gathered on lipid metabolites and EST profiles: A. Fatty acid biosynthesis and chain termination, B. Glycerolipid synthesis in plastids, C. Glycerolipid synthesis in the ER, D. Synthesis of phospholipids via the CDP-DAG pathway in the ER. The number of ESTs in elongating fiber (7 to10 dpa), predicted to encode for the different enzymes, are shown in parentheses. Enzymes for which no ESTs were identified are marked with an asterisk. The EST data was obtained from The Institute for Genomic Research through the Website at http://www.tigr.org and cataloged in table 1.

Nine ESTs were found to be highly homologous with sequences of known acyl-CoA acyltransferases. In *A. thaliana*, only 2 ESTs for each of these enzymes have been identified. Transfer of a second acyl chain onto LPA forms phosphatidic acid (PA), and several ESTs were annotated as encoding the plastidial isoform for LPA acyltransferase while several others were annotated as encoding the ER isoform. The presence of all these ESTs suggest that synthesis of PA, which is a major precursor for most glycerolipid synthesis, occurs in both the plastid and ER of fiber cells. The remaining cotton fiber ESTs annotated as encoding for acyltransferases did not show high homology with any specific isoform and may be orthologs encoding for enzymes involved in similar reactions, or other unidentified acyltransferases.

Phosphatidic acid (PA) serves as the precursor to most glycerolipids synthesized via the nucleotide pathway, which uses diacylglycerol (DAG) or the cytidine diphosphate:diacylglycerol (CDP: DAG) pathway (Kinney,1993). PA may be dephosphorylated by phosphatidic acid phosphatase forming DAG, and a single EST predicted to encode for this enzyme was present, while 4 ESTs were annotated as encoding for DAG kinase, which catalyzes the reverse reaction. DAG is used in the synthesis of galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which is thought to occur in plastids and is catalyzed by MGDG synthase and DGDG synthase respectively (Joyard et al., 1993), but no ESTs were annotated as encoding for these enzymes. DAG may also be acylated by DAG acyltransferase (DAGAT) to form triacylglycerides (TAGs), but no ESTs were identified for this enzyme, nor were any ESTs identified as encoding for phospholipid diacylglycerol acytransferase (PDAT), which catalyzes the transfer of an acyl group

from a phospholipid to DAG forming TAG and a lysophospholipid. These data suggest that TAG which serve mainly as a storage reserve, is not synthesized in the early stages of fiber development.

In the ER, DAG acts as a precursor for synthesis of major membrane phospholids in the ER via the action of a phosphotransferase, displacing cytidine monophosphate(CMP) form the aminoalcohols CDP-choline or CDP ethanolamine, to synthesize phoshatidylcholine (PC) and phosphatidylethanolamine (PE), respectively (Kinney, 1993). The aminominoalcohols are themselves produced in a series of reactions in which the head groups, choline and ethanolamine are first phosphorylated, and then reacted with cytidine triphosphate (CTP) (Kinney, 1993). A BLAST search against the Arabidopsis genome, revealed 3 ESTs that could be annotated as encoding for choline kinase, which phosphorylates choline to phosphocholine. While no ESTs were identified as encoding for ethanolamine kinase it is possible that the same enzyme catalyzes the phosphorylation of both choline and ethanolamine. Similarly, a single cytidyltransferase enzyme is thought to use either phosphocholine or phosphoethanolamine as a substrate is the next reaction in the series, and a single aminoalcohol phosphotransferase catalyzes the final step producing PC or PE (Goode and Dewey, 1999). Four ESTs were annotated as encoding for the cytidyltransferase enzyme that catalyzes the reaction with CTP to form CDP-choline and CDP-ethanolamine, while 2 ESTs were predicted to encode for the aminoalcohol phosphotransferase (AAPT).

In plastids PA may also be converted to CDP: DAG by action of CDP: DAG synthase, but no ESTs encoding for this enzyme, were identified. CDP: DAG is thought

to a precursor for synthesis of plastidial phospholipids such as phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) (Joyard et al., 1993). PGP synthase, which uses CDP: DAG and G3P to form PGP is the precursor to PG and 2 ESTs were predicted to encode for the plastidial isoform of PGP synthase, while a single EST in the 6dpa library was annotated as encoding for the ER isoform. However, no ESTs were identified as encoding for PGP phosphatase, which produces PG form PGP. Additionally, no ESTs were predicted to encode for PI synthase or PS synthase, which both use CDP: DAG as a precursor. These results suggest that these enzymes may have rare messages, not yet identified in these libraries, or that these phospholipids are produced via alternative pathways in cotton fiber cells.

Desaturation of acyl chains of phospholipids occurs in both the plastid and ER and is catalyzed by different proteins in these organelles. Ten ESTs predicted to encode for the ER oleate desaturase (fad 2), was identified, while only a single EST was annotated as encoding for the plastidial isoform fad 6. Five ESTs were annotated as encoding the ER linoleate desaturase (fad 3), while 8 ESTs were predicted to encode for the plastidial isoform fad 7/fad 8.

The large number of ESTs predicted to encode for enzymes involved in fatty acid elongation and wax biosynthesis indicates that elongating fiber cells are actively engaged in these processes. The fatty acid elongase system, which adds 2 carbons per cycle to C18-acylCoAs, consists of four components; β-ketoacylCoA synthase, β-ketoacylCoA reductase , β-hydroxylacyl-CoA dehydratase and enoyl-CoA reductase (Somerville et al., 2000). A large number of ESTs (150) were identified as encoding for β-ketoacylcoA synthase, while15 ESTs were annotated as encoding for the reductase,

while no ESTs were identified for the latter two components of the elongase system. The highest numbers of ESTs predicted to encode for a single enzyme were those for lipid transfer protein (LTP) precursors. A total of 218 ESTs in the 7 to 10dpa library and 379 ESTs in the 6dpa library were annotated as encoding for this protein. LTPs are thought to have a role in cutin deposition and fiber specific transcripts of this enzyme have been identified in cotton and found to be highly expressed during the stage of rapid elongation (Orford and Timmis, 2000, Ma et al., 2000).

Sphingolipids form a small percent of membrane lipids and several ESTs for enzymes involved in their biosynthesis were identified in the cotton fiber library. Four ESTs were annotated as encoding for serine palmitoyltransferase, which condenses a serine and palmitoyl-CoA forming 3-ketosphinganine, in the first step of the pathway (Lynch, 1993). Several ESTs were identified for 3-ketosphinganine reductase which forms sphinganine, the precursor to ceramides. Ceramide synthesis is thought to occur either by acylation of sphinganine with a fatty acid, or using an acyl-Co A donor (Lynch, 1993). Ceramide synthase catalyzes the former reaction and a single EST was identified for this enzyme, while no ESTs were annotated as encoding for sphinganine acyltransferase, which catalyzes the latter reaction. A single EST predicted to encode for a sphingolipid desaturase was identified.

Sterols have been found to be minor components of cotton fiber (Amin and Truter, 1972), and several ESTs encoding enzymes involved in sterol biosynthesis were identified in the cotton fiber libraries. These include 21 ESTs for HMGCoA reductase, a major precursor in sterol biosynthesis, 14 ESTs for 24 sterol methytransferase, 2 ESts for sterol C14 reductase, 2 ESTs for the delta 8 delta 7 isomerase, 5 ESts for sterol-4

alpha methyl oxidase, 6 ESTs for UDP: glucose sterol glucosyltransferase and 8 ESts for SAM Sterol C- methyltransferase. The occurrence of these ESTs is interesting because of published reports of sterol glycosides as precursors of cellulose biosynthesis (Peng et al., 2002).

Plant lipids undergo remodeling during growth and development as well as in response to various environmental conditions and lipases are involved in these processes. Phospholipase D (PLD) is known to hydrolyze phospholids producing phosphatidic acid and a head group, and different isoforms of this enzyme have been identified (Wang, 2000). In the cotton fiber libraries, the most abundant ESTs were those for PLDα, for which 25 ESTs were identified while only 2 ESTs were identified as encoding for PLDβ and a single EST was identified for the PLD γ isoform. Several ESTs (84) were grouped as homologs of lysophospholipases , which themselves hydrolyze phospholids producing lysophospholipids and a free fatty acid . Two ESTs were specifically annotated as encoding for phospholipase A1, which cleaves phospholipids at the sn-1 position, while 7 ESTs were predicted to encode for phospholipase A2, which cleaves phospholipids at the sn-2 position. Additionally, 12 ESTs were annotated as encoding for PI specific phospholipase C which hydrolyzes PI into DAG and inositol phosphate, both well characterized signaling molecules in cells.

Fatty acids in the form of acyl-CoAs are degraded via β-oxidation. AcylCoA oxidase, oxidizes fatty acyl-CoAs, producing 2-transenoyl-Co A, which is hydrated, by enoyl-CoA hydratase, forming 3-hydroxylacylCoA, that is subsequently oxidized to 3-oxoacyl-CoA. 3-KetoacylCoA thiolase, then hydrolyzes the oxoacylCoA producing a fatty acyl-CoA with 2 fewer carbons, and acetyl-CoA (Somerville et al., 2000). Several

ESTs for each of the enzymes involved in this process were present in the cotton fiber library. These included 4 ESTs for acyl-CoA oxidase, 7 ESTs for enoylCoA hydratase, 3 ESTs for 3-hydroxybutyryl-CoA dehydrogenase, 5 ESTs for 3- KetoacylCoA thiolase and 8 ESTs for Acetyl-CoA acyltransferase. The expression of these genes in developing cotton fiber suggests that fatty acid degradation occurs even at the early stages of fiber development.

CHAPTER 4

DISCUSSION

Cotton fibers are single cells that develop relatively synchronously, forming extremely long cells in a period of a few days hence they represent a good system for studying cell growth. The rapid elongation of these cells requires substantial lipid synthesis to support the developing organelle and membranes. Expression profiles of genes in developing cotton fiber cells suggested that lipid metabolism occurs in these cells. The presence of several ESTs for enzymes involved in fatty acid biosynthesis indicated that this pathway is fairly active at the early stage of fiber development. A large number of ESTs were predicted to encode for ACCase, which is considered to be the major regulatory enzymes in the pathway (Post-Beittenmiller et al., 1991). Several ESTs were identified for ACPs , which carry the fatty acids through the assembly process, as well as for KAS I which is responsible for fatty acyl chain elongation, producing carbon acyl chains. Glycerolipid synthesis usually begins with incorporation of 16:0 and 18:1 acyl chains onto a glycerol 3-phosphate backbone. KAS II is responsible for the elongation of 16:0 to stearate (18:0) and we hypothesized that some of the ESTs grouped as encoding for KAS I may in fact be KAS II and KAS III ESTs, due to the high sequence homology. Several ESTs for Stearoyl-ACP desaturase, that introduces the first double bond in stearate (18:0) forming oleate (18:1), were prevalent as well. These EST profiles indicate that cotton fiber cells are capable of producing a substantial amount of 16:0 and 18:1, and these fatty acids are then incorporated into complex lipids during plastidial and extraplastidial glycerolipid synthesis.

The lipid composition in plant membranes is important for membrane function, as membranes need remain fluid to allow membrane proteins to function properly. The fatty acid composition is highly regulated in plants especially the ratio of saturated to unsaturated acyl chains. The fatty acid profiles of cotton fiber cells were found to remain fairly constant throughout the different stages of fiber development. The fatty acid composition at the 7dpa, 14dpa, 21dpa and 28dpa showed an abundance of the polyunsaturated fatty acid linolenate (18:3) while the saturated fatty acid palmitate (16:0) was fairly prevalent as well. Linoleate (18:2) and oleate (18:1) were less prevalent, and stearate (18:0) made up only a small percent of total fatty acids. Palmitate (16:0), the predominant saturated fatty acid in most plants, is a major product of de novo fatty acid biosynthesis (Ohlrogge, 1993), while 18:3 arises from desaturation of 18 carbon acyl chains esterified to phospholipids. Regulating membrane lipid composition by desaturation of acyl chains plays an important role in ensuring that membrane fluidity is maintained, and may explain why such an abundance of 18:3 was observed in cotton fibers. Saturated fatty acids such as 16:0 and 18:0 are important for membrane stability, or may be elongated to very long chain fatty acids which serve as precursors to biosynthesis of surface components such as epicuticular wax (Post-Beittenmiller, 1996). EST profiles, showing an abundance of ESTs for fatty acid elongation and wax biosynthesis, indicated that fibers are actively involved in these processes and would require the necessary acyl chain precursors.

Glycerolipid synthesis occurs in both plastids and ER of cells, and several ESTs involved in this process were identified. Profiles of ESTs in elongating cotton fibers provide support for synthesis of PA, which serves as a precursor to most glycerolipids.

PA when dephosphorylated to DAG may be used for synthesis of PC and PE as well as the galactolipids MGDG, DGDG and SQDG. Alternatively, PA serves as a precursor to CDP: DAG synthesis, which in turn is used to produce PG, PI and PS. The polar lipid composition of developing and mature cotton fibers showed that PC was the most abundant lipid class, while PI and PE were also fairly prevalent. PC has been found to be a major membrane lipid from various compartments except plastids (Ohlorogge and Browse, 1995). PS was found in very relatively low quantities, as were the plastidial lipids PG, MGDG and DGDG.

The composition of membrane lipids derived from PA may provide some insight into the pathways operating within fiber cells. PA produced via the prokaryotic pathway, has 16:0 esterified at the sn-2 position of glycerol 3-phosphate, while that produced via the eukaryotic pathway has 18-carbon fatty acids at the sn-2 position and where it occurs, 16:0 is found esterified at the sn-1 position (Frentzen, 1993). The molecular species composition revealed that for PC as well as the other major membrane phospholipids PI and PE, 34:3(18:3,16;0) and 36:6(18:3,18:3) were the predominant species. PC is known to be a substrate for acyl lipid desaturases, and EST profiles showed several oleate desaturase and linoleate deasaturase ESTs, which would also explain the abundance of 18:3 fatty acids in the membrane.

PC may serve as a precursor to synthesis of galactolipids found in plastidial membranes such as MGDG and DGDG (Ohlrogge and Browse, 1995). Analysis of the molecular species of these galactolipids in cotton fiber cells revealed that 36:6 (18:3,18:3) was the predominant species. This composition with 18 carbon fatty acids at the sn-2 position, suggests a eukaryotic origin, hence the DAG from which these lipids

were synthesized was most likely obtained from PC in the ER and translocated to the plastid. The molecular species composition of PG in fiber cells, with an abundance of 32:0(16:0,16:0), 32:1(16:0,16:1) and 34:3(16:0,18:3), suggests that unlike MGDG and DGDG, PG, is synthesized via both the prokaryotic as well as the eukaryotic pathway. In many higher plant species, PG is the only lipid that is synthesized via the prokaryotic pathway, while galactolipids synthesis via this pathway occurs only in certain plants, termed 16:3 plants due to the accumulation of this fatty acid in their leaf tissue (Wallis and Browse, 2000). PA synthesized in the plastid envelope produces DAG via a membrane bound PA phosphatase, but the low activity of this enzyme is not sufficient to support synthesis of galactolipids , and explains why 18:3 plants contain only small amounts of galactolipids with 16 carbon fatty acids at the sn-2 position, but contain PG with this structure (Joyard et al., 1993).

The high levels of PA observed in frozen cotton fiber cells, were unusual and we postulated that this may due to hydrolysis of phospholipids mediated by PLD, that occurs post harvest, due to a freezing and thawing of fiber cells. EST profiles show several ESTs for the PLD and in particular for the PLD α isoform. Preliminary PLD assays on cotton fiber have shown that this enzyme is highly active and readily hydrolyzes radioactive PC into PA. Transphospatidylation, which is characteristic of PLD activity, was also evident in these cotton fiber assays.

In some cases EST profiles were insufficient to explain the synthesis of lipid metabolites observed in our biochemical analysis. Synthesis of phospholipids produced via the CDP: DAG pathway was not well supported by EST profiles. No ESTs were identified for the CDP: DAG synthase which uses PA to produce CDP: DAG the main

precursor of PG, PI and PS produced in this pathway. However, a few ESTs were annotated as encoding for plastidial PGP synthase which uses CDP: DAG and G3P as precursor for PGP synthesis, but no ESTs were identified for PGP phosphatase which is necessary for the production of PG. It is noteworthy that no candidate genes for this enzyme have been identified in *A. thaliana* (Beisson et al., 2003). In addition, no ESTs were predicted to encode for PI synthase which is especially surprising as an abundance of PI is observed in both elongating and maturing cotton fiber cells. PS was found in relatively low quantities, as compared to other phospholipids, but no ESTs were annotated for PS synthase. No candidate genes have identified in *A. thaliana* for PS synthase (Beisson et al., 2003) nor have any been identified for PE: L-serine phosphatidyltransferase, which allows for synthesis of PS by exchange of serine with the head group of PE (Kinney, 1993).

Metabolic schemes for synthesis of fatty acids and glycerolipids in cotton fiber cells were developed based on the data gathered on lipid metabolites and EST profiles. The results obtained raise questions as to the mechanisms by which some of the lipid metabolites are produced in cotton fiber cells, and further studies are needed to provide clarification. Taken together the results presented in this thesis will provide the basis for future studies aimed at developing models for metabolic regulation of membrane lipid metabolism in these unusual fiber cells. It is possible that a better understanding of cotton fiber membrane biogenesis will lead to new strategies to improve the qualities of this important agricultural commodity.

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