Identification and Quantification of Glycerolipids in Cotton Fibers: Reconciliation with Metabolic Pathway Predictions from DNA Databases

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ABSTRACT: The lipid profiles of cotton fiber cells were determined from total lipid extracts of elongating and maturing cotton fiber cells to see whether the membrane lipid composition changed during the phases of rapid cell elongation or secondary cell wall thickening. Total FA content was highest or increased during elongation and was lower or decreased thereafter, likely reflecting the assembly of the expanding cell membranes during elongation and the shift to membrane maintenance (and increase in secondary cell wall content) in maturing fibers. Analysis of lipid extracts by electrospray ionization and tandem MS (ESI-MS/MS) revealed that in elongating fiber cells (7–10 d post-anthesis), the polar lipids—PC, PE, PI, PA, phosphatidylglycerol, monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and phosphatidylglycerol—were most abundant. These same glycerolipids were found in similar proportions in maturing fiber cells (21 dpa). Detailed molecular species profiles were determined by ESI-MS/MS for all glycerolipid classes, and ESI-MS/MS results were consistent with lipid profiles determined by HPLC and ELSD. The predominant molecular species of PC, PE, PI, and PA was 34:3 (16:0, 18:3), but 36:6 (18:3,18:3) also was prevalent. Total FA analysis of cotton lipids confirmed that indeed linolenic (18:3) and palmitic (16:0) acids were the most abundant FA in these cell types. Bioinformatics data were mined from cotton fiber expressed sequence tag databases in an attempt to reconcile expression of lipid metabolic enzymes with lipid metabolite data. Together, these data form a foundation for future studies of the functional contribution of lipid metabolism to the development of this unusual and economically important cell type.

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Abbreviations: ACP, acyl carrier protein; ASG, acylated sterol glycoside; CDP, cytidine diphosphate; CMP, cytidine monophosphate; DGDG, digalactosyldiacylglycerol; dpa, days post-anthesis; ER, endoplasmic reticulum; ESI, electrospray ionization; EST, expressed sequence tag; FAD, fatty acid desaturase; G3P, glycerol-3-phosphate; KAS, ketoacyl ACP synthase; KLRC, Kansas Lipidomics Research Center; LPA, lysophosphatidic acid; LPC, lysoPC; LPE, lysoPE; LPG, lysoPG; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PLD, phospholipase D; SG, sterol glycoside; TC, tentative consensus. The numerical designation of acyl groups is represented as number of acyl carbons:number of double bonds.

Cotton fibers are trichomes that arise from the differentiation of ovular epidermal cells near or on the day of anthesis (1). Development of these cells progresses through stages of elongation and secondary wall deposition forming extremely long single cells, over an inch (2.5 cm) long (2), during a period of about 25 d. This period of rapid cellular elongation requires substantial synthesis of macromolecules, including lipids necessary for the developing vacuoles and plasma membranes. The major research focus on cotton fiber development historically has been on cellulose synthesis, which accounts for the bulk of the mass of mature fiber cells; however lipid metabolism is likely to be an important factor in the development of cotton fibers, and this subject has received little attention.

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 (3,4). This is followed by elongation, in which the cells undergo rapid expansion with increases in length and diameter, and concurrent primary cell wall formation. On average, this continues for 15–27 d (3). Secondary cell wall synthesis begins around 16–19 d post-anthesis (dpa) and is thought to overlap with elongation (5). This process consists of the deposition of successive layers of cellulose microfibrils in a helical pattern around fiber cells (2,6). Maturation of fibers ends about 45–60 dpa, and it includes seed capsule dehiscion, dehydration, and collapse of fiber cells (2).

Synthesis of cellular constituents, including lipids for incorporation into the vacuole and plasma membranes, must be necessary during the period of rapid cell expansion in cotton fiber cells. In plants, FA biosynthesis occurs *de novo* in plastids. The newly synthesized FA can be either incorporated into plastidial glycerolipids or transported to the endoplasmic reticulum (ER) for incorporation into extraplastidial complex lipids (7) such as those destined for vacuole and plasma membranes. Developing fiber cells, undergoing rapid elongation, have a higher lipid content and were shown to incorporate the majority of ^{14}C-labeled acetate into polar lipids at a rate that declines as cell elongation ceases [from 10 to 20 dpa (2)]. As polar lipids are the major constituents of lipid bilayers, these results suggest that fiber cells are involved in active membrane synthesis during early stages of development.

Recent technical advances now make it possible to perform rapid, detailed glycerolipid profiling (8). Electrospray ionization

(ESI) and tandemmass spectrometry (MS/MS) are currently used to determine the classes, molecular species, and amount of lipids in plant cells (8). Here we have applied this technique to identify and quantify the classes and molecular species of polar lipids in developing cotton fiber cells. These data provide a critical knowledge base of information for how lipid composition changes with fiber development. The results were compared with those from other approaches, including GC-FID, to determine total FA composition, and HPLC analysis combined with ELSD or MS to profile polar lipids as well as sterols, sterol esters, and sterol glycosides (SG) (9,10). SG and their acylated components in cotton fiber cells have been implicated as primers for elongation of β1–4 glucan chains that form cellulose microfibrils during secondary cell wall development (11); hence the sterol composition of cotton fiber cells may be important to understanding more about cell wall formation in these fiber cells.

Several genes that are preferentially expressed in cotton fibers during the rapid elongation process have been identified, including some that are involved in lipid metabolism, such as those encoding a putative acyltransferase and a putative 24-sterol-C methyltransferase (12). Transcripts for a gene encoding a fiber-specific acyl carrier protein (ACP) were highly expressed during the stage of elongation, and this ACP was proposed to play a role in synthesis of membrane lipids (13). Cotton fiber-specific cDNA encoding lipid transfer proteins have been isolated and shown to have a temporal expression pattern, accumulating from 6 to 14 dpa (14,15); transcript levels for one isoform, lipid transfer protein-3, reached a maximum at 15 dpa (16). The results of these studies indicate that transcription of genes involved in lipid metabolism in cotton fiber occurs in a developmentally regulated manner. However, a more global examination of gene expression in cotton fiber cells during the stage of rapid elongation might provide more insights into the mechanisms responsible for the development of these cells.

More than 50,000 expressed sequence tags (EST) from *Gossypium* sp. fiber cells have been sequenced by several laboratories [predominantly Brookhaven National Laboratory (Brookhaven, NY) and Clemson University Genomics Institute (Clemson, SC)] and compiled by The Institute for Genomic Research (TIGR; Rockville, MD) at their web site at http://www.tigr.org. Most all of these EST are derived from cotton fibers at 6 or 7 to10 dpa, during the stage of rapid cell elongation, and most likely include expressed genes of lipid metabolism important for this development stage. The occurrence of EST 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, as was done recently for oil biosynthesis in *Arabidopsis* seeds (17). The combination of metabolic profiles and DNA database information provides a unique perspective on membrane biogenesis for fiber cell elongation and will serve as a basis for the development of hypotheses regarding the regulation of membrane biogenesis in this important agricultural plant.

The main objective of this research was to characterize the glyceroplipid metabolites in cotton fiber cells at different stages of development and estimate a gene expression profile for lipid metabolism genes in elongating cotton fibers. Toward this end we compiled a list of EST for enzymes involved in lipid metabolism in developing cotton fibers, using EST data annotated and assembled into tentative consensus (TC) sequences by The Institute for Genomic Research. Unique TC sequences are estimates of individual transcripts in cases such as cotton where the genome has not yet been sequenced. The data on EST profiles were viewed, compared, and correlated with the levels of various lipids (including both lipid classes and their individual molecular species) and were used to predict lipid metabolic pathways in developing cotton fiber cells.

MATERIALS AND METHODS

Plant material. Cotton (*Gossypium hirsutum* L. cv. Coker 312) plants were greenhouse grown in April to October in Denton, Texas, with day/night temperatures of 95/80°F (35/27°C) and an approximate 16 h photoperiod (day length extended with sodium vapor lamps when necessary). Flowering plants were tagged on the day of anthesis, and bolls were harvested at 7–10, 14, 21, and 28 dpa. Material in locules (fiber and ovules) was excised and immediately placed in boiling isopropanol, since experiments in which samples were kept on ice or stored at –20 or –80°C resulted in large amounts of post-harvest-generated PA. Replicate fiber samples were dried overnight at 50°C to determine dry weight of fiber samples. Dry weights ranged from 45 to 100 mg. Total FA profiles also were determined for cv. Stoneville 474 (SG474), grown in Stoneville, Mississippi, over the same period for comparison. In this case, bolls were provided by Dr. Jodi Scheffler, USDA-ARS, Cotton Physiology and Genetics (Stoneville, MS).

Chemicals. Diheptadecanoyl (17:0) L-α-PC, L-α-PC (Type II) from soybean, cabbage phospholipase D (PLD) (Type V), and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Dipalmitoyl-L-α-PC was purchased from NEN Life Sciences (Boston, MA). All other chemicals were from Fisher Scientific unless otherwise stated. Phospholipid and galactolipid standards for ESI-MS/MS were obtained and quantified as previously described (8).

Lipid extraction and analysis. Total lipid extraction of cotton tissues was by a modified version of the Bligh/Dyer method using isopropanol and chloroform (as described in Ref.18). Approximately, 0.5 g of fiber samples was placed in 2 mL boiling isopropanol (70°C). L-α-Diheptadecanoyl (17:0) PC (1–5 mg) was added to some samples as a quantitative standard, and the tubes were incubated at 70°C for 30 min. The samples were allowed to cool to room temperature, 1 mL of chloroform was added, and the samples were extracted overnight at 4°C. Following overnight extraction, phase separation was achieved by addition of 2 mL of KCl and 1 mL of chloroform, and facilitated by centrifugation at 480 $-\times$ *g* for 10 min in a Beckman TJ-6 benchtop centrifuge. The upper, aqueous phase was aspirated, and the remaining organic layer was washed two times with 2 mL 1 M KCl and finally with 1 mL ultrapure water

(Milli-Q). The chloroform was evaporated using a Multivap118 nitrogen evaporator (Organomation Associates, Berlin, MA), and 2 mL of fresh chloroform was added to the remaining lipid extract. The lipid samples were stored under nitrogen at –20°C for subsequent FA analysis, or evaporated to dryness and shipped overnight under nitrogen to the Kansas Lipidomics Research Center (KLRC) Analytical Laboratory for ESI-MS/MS analysis, or to the Eastern Regional Research Laboratory, USDA, for HPLC-ELSD analysis.

For total FA analysis, the lipid extracts were evaporated to dryness with nitrogen gas and transesterified in 1 mL acidic (1% H_2SO_4) methanol for 30 min at 65°C. FAME were extracted into hexanes, washed with 5% (wt/vol) NaCl, and dried over Na_2SO_4 columns. FAME in hexanes were analyzed by GC-FID using a Hewlett-Packard Series II plus 5890 gas chromatograph. Samples were separated over a 30 m SUPELCOWAX^{TM}-10 fused-silica capillary column (Supelco, Bellefonte, PA) at an oven temperature of 200°C, with nitrogen as the carrier gas, and FID was used to quantify FAME. Injector and detector temperatures were 250°C. Identification of FA in samples was achieved by comparison with lipid standards (FAME Mix GLC–10; Supelco), and the amount of each FA was calculated based on the amount of the internal heptadecanoyl standard (from the transesterification of diheptadecanoyl PC).

The polar lipid classes and molecular species in cotton fiber lipids were determined by direct infusion ESI and MS/MS at the KLRC Analytical Laboratory. The samples were dissolved in chloroform. Two aliquots were taken for MS analysis, one sample of 10 μL for phospholipid analysis and one of 10 μL for galactolipid analysis. For phospholipid analysis, cotton lipid extract in chloroform was combined with solvents and internal standards, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300:665:35, the final volume was 1 mL, and the sample contained 0.66 nmol di14:0-PC, 0.66 nmol di24:1-PC, 0.66 nmol 13:0-lysoPC, 0.66 nmol 19:0-lysoPC, 0.36 nmol di14:0-PE, 0.36 nmol di24:1-PE, 0.36 nmol 14:0-lysoPE, 0.36 nmol 18:0-lysoPE, 0.36 nmol di14:0-phosphatidylglycerol (PG), 0.36 nmol di24:1-PG, 0.36 nmol 14:0-lysoPG, 0.36 nmol 18:0-lysoPG, 0.36 nmol di14:0-PA, 0.36 nmol di20:0(phytanoyl)-PA, 0.24 nmol di14:0-PS, 0.24 nmol di20:0(phytanoyl)-PS, 0.20 nmol 16:0–18:0-PI, and 0.16 nmol di18:0-PI. For galactolipid analysis, plant lipid extract in chloroform was combined with solvent and internal standards, such that the ratio of chloroform/methanol/50 mM sodium acetate in water was 300:665:35, the final volume was 1 mL and the sample contained 2.01 nmol 16:0–18:0-monogalactosyldiacylglycerol (MGDG), 0.39 nmol di18:0-MGDG, 0.49 nmol 16:0–18:0-digalactosyldiacylglycerol (DGDG), and 0.71 nmol di18:0-DGDG.

The samples were analyzed on a triple quadrupole tandem mass spectrometer (API 4000; Applied Biosystems, Foster City, CA) equipped for ESI. The source temperature (heated nebulizer) was 100°C, and -4.5 or +5.5 kV was applied to the electrospray capillary. The two ion source gases were each set at 45 arbitrary units; the curtain gas was set at 20 arbitrary units; the declustering potential was ±100 V for phospholipids, +150 V for MGDG, and +215V for DGDG; and the entrance potential was ±10 to ±15 V. With the interface heater on, the sample was introduced using an autosampler (LC Mini PAL; CTC Analytics AG, Zwingen, Switzerland) and presented to the ESI needle at 30 μL/min. Precursors of lipid head-group-derived fragments were detected, using the scans previously described (8,19,20). The collision energies, with nitrogen in the collision cell, were 28 V for PE, 40 V for PC, −58 V for PI, −57 V for PA and PG, −34 V for PS, 50 V for monogalactosyldiacylglycerol (MGDG), and 84 V for digalactosyldiacylglyerol (DGDG). The mass analyzers were adjusted to a resolution of 0.7 amu full width at half height. For each spectrum, 9–150 continuum scans were averaged in multiple channel analyzer mode.

Data processing was performed using Analyst software (Applied Biosystems) with a custom script written by Anna Lisyansky of Applied Biosystems. In each spectrum, the background was subtracted, the data were smoothed, and then peak area was determined. Identification of the peaks of interest and calculation of lipid species amounts were performed using Microsoft Excel. Corrections for overlap of isotopic variants (A + 2 peaks) in higher mass lipids were applied. The lipids in each class were quantified in comparison with the two internal standards of that class as described (8).

For the quantitative analysis of the polar and nonpolar lipid classes, the total lipid extract was dissolved in chloroform/methanol (10 mg/mL in 85:15, vol/vol) and injected in an HPLC system equipped with ELSD. The method used an Agilent 1100 HPLC system, a Sedex Model 55 ELSD, and a DIOL column, as previously described (10). Lipid classes were identified by cochromatography with commercial standards and by MS (LC-MS with atmospheric pressure chemical ionization in the positive ion mode).

Bioinformatics and data mining. Cotton EST from different tissues in cotton have been assembled into TC sequences at The Institute for Genomic Research web site at http://www.tigr.org. These include EST from two libraries for cotton fibers at 7–10 dpa and at 6 dpa, which is the stage of rapid cell expansion. We queried these databases to catalog EST to identify the major enzymes involved in lipid metabolism in elongating cotton fiber. At the time of our analysis 38,814 EST were annotated from the 7–10 dpa library, and 8,003 from the 6 dpa library. Hence, the number of EST and TC sequences discussed here are almost exclusively those of the larger (7–10 dpa) library, with certain noted exceptions. Some EST were already annotated, whereas others were identified using the WU-BLAST program with cDNA and protein sequences from other plants as queries. Many of these plant sequences were obtained from a lipid gene database for *Arabidopsis thaliana* housed at http://www.plantbiology.msu.edu/lipids/genesurvey/index.htm (17). In addition, protein motif identification, domain organization analysis, and subcellular location predictions were accomplished with web-based bioinformatics tools (interpro, BLOCKS, NCBI-DART, PSORT, TargetP) where possible. Sequences of lipid metabolism genes in cotton were placed in the context of known lipid metabolism pathways and used to predict

the metabolic capacity for cotton fibers to produce various lipid metabolites. Actual metabolite data were compared with these metabolic schemes to provide an overall view of lipid metabolism in elongating cotton fiber cells.

All experiments were repeated at least three times and the data presented are the means ± SD.

RESULTS

FA composition of cotton fiber cells. Total lipid extracts were obtained from cotton fiber cells (*G. hirsutum*, L., cv. SG474 and Coker 312) harvested at different ages (7, 14, 21, and 28 dpa) representative of different stages of fiber development. Total FA composition was determined by GC-FID using heptadecanoic acid (17:0) as a quantitative internal standard. The percentage by weight of each of the common FA, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3), remained relatively constant over the entire period of fiber development (Fig. 1). Linolenic was found to be the most abundant FA, accounting for about 40 to over 50% by weight of total FA. Palmitic was the second-most abundant FA, representing 25–35% by weight of total FA. Linoleic acid constituted 10–15% by weight of FA present, whereas low levels of oleic and even lower levels of stearic acid were observed in all stages of development. A slight increase in the ratio of saturated to unsaturated FA was observed in the later stages of fiber development.

The total amounts of individual FA increased (or were highest) during the elongation stage and decreased thereafter (Fig. 2), despite a relative increase in the size of bolls and fiber content during fruit development. During the early stages of fiber development, cells are undergoing rapid elongation (2) and are engaged in lipid synthesis for membrane development; hence, an increase in fiber mass in bolls is associated with a concomitant increase in lipid content. In maturing fibers, cells are involved in secondary cell wall synthesis, which begins around 16–19 dpa and continues up to 25 dpa (2). During this stage of development, cellulose accumulates 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 lower in maturing fibers. The slight difference in the time course of lipid accumulation between the two cultivars may be genetic or may be environmental; however, the trends are the same, with lipid accumulating early to support cell elongation, and then tapering off as fiber cell wall thickening proceeds. Cotton embryos begin to synthesize seed oil at around 20–25 dpa. Hence, membrane lipid synthesis in fiber cells may decline prior to this period of TAG accumulation during cottonseed development.

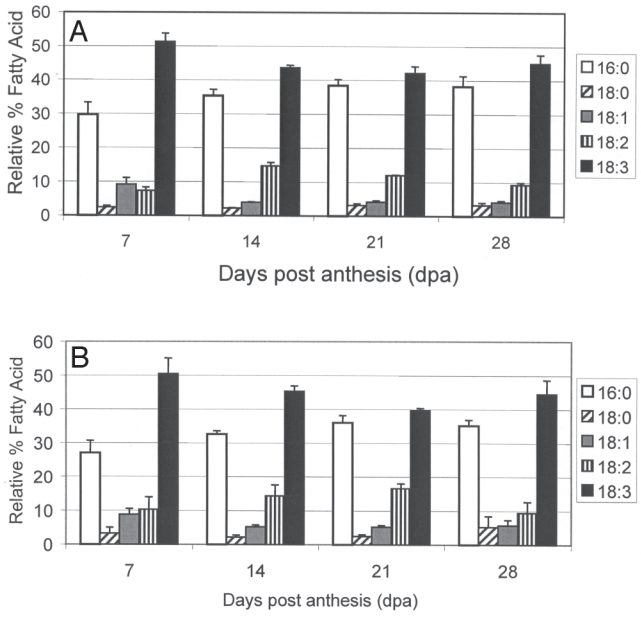

FIG. 1. The FA composition of cotton fiber cells at different stages of development [7, 14, 21, and 28 d post-anthesis (dpa)] was determined for two different cultivars–*Gossypium hirsutum* L. cv. Stoneville SG474, upper panel, and cv. Coker 312, lower panel. Total lipids were extracted from fiber cells with addition of diheptadecanoyl (di17:0) PC as an internal standard. Following acid-catalyzed transesterification, total FAME were analyzed by GC-FID and quantified based on the amount of methyl heptadecanoate (17:0) from the internal standard. The relative proportions (wt %) of the common FA present in SG474 fibers and Coker 312 fiber cells were compared. The overall composition was similar for both cultivars throughout the entire period of fiber growth and maturation, and 18:3 and 16:0 were the most abundant FA at each stage of development. Bars represent the means and SD of three (SG474) or four (Coker 312) independent extractions.

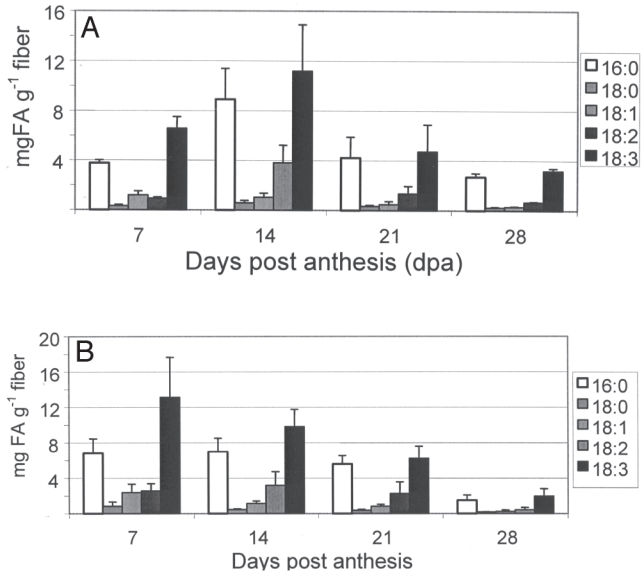

FIG. 2. Individual FA content was quantified (mg per dry weight of fiber) at the different stages of fiber development. In SG474 (A), an increase in 16:0, 18:2, and 18:3 content occurred from 7 to 14 dpa (during cell elongation), with levels declining on a cell dry weight basis thereafter (during maturation). For Coker 312 (B), the levels of individual FA were highest at 7 and 14 dpa (during cell elongation), and similar to SG474. All FA decreased on a cell dry weight basis at 21 and 28 dpa (during maturation). Bars represent the means and SD of three (SG474) or four (Coker 312) independent extractions.

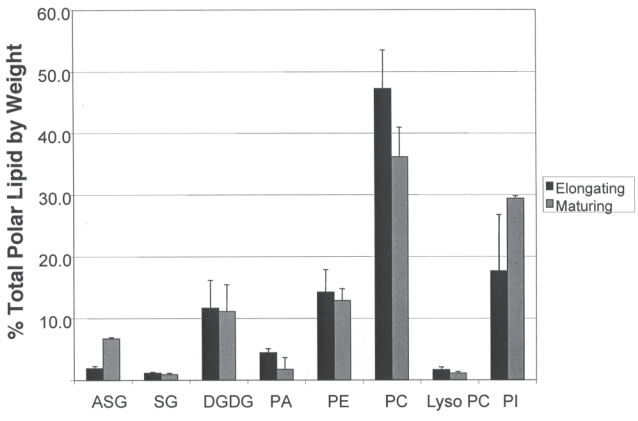

FIG. 3. Comparison of polar lipid classes of elongating (8–10 dpa) and maturing (21 dpa) fiber cells (from cv. Coker 312) determined by HPLC-ELSD and expressed as weight percentage of the total polar lipid (10). Lipids were quantified based on standard curves developed for each lipid class. ASG, acylated sterol glycoside; SG, sterol glycoside; DGDG, digalactosyldiacylglycerol (may include MGDG, monogalactosyldiacylglycerol); PA, phosphatidic acid; LPC, lysophosphatidylcholine. Bars represent the means and SD of three independent extractions.

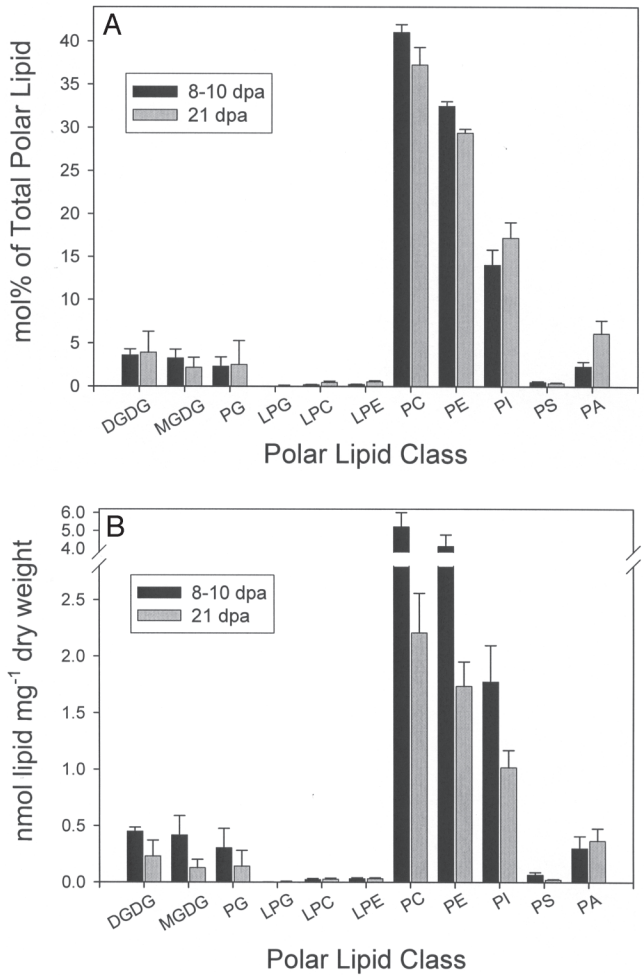

FIG. 4. Comparison of glycerolipid content of elongating (8–10 dpa) and maturing (21 dpa) fiber cells (from cv. Coker 312) determined by ESI-MS/MS. In panel A lipid quantities are presented as mol% of total glycerolipid, and in panel B lipids are quantified on nmol mg^{-1} dry weight basis. Lipid quantities were calculated based on internal standards included for each class (see methods) and the amount for each class represents the sum of individual molecular species for that class. PG; phosphatidylgycerol; LPG, lysophosphatidylglycerol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid. Bars represent the means and SD of five independent extractions.

Polar lipid profiles in cotton fiber cells. The polar lipid classes of elongating and maturing cotton fiber cells were quantitatively analyzed by HPLC-ELSD analysis (Fig. 3). PC was the most abundant lipid class, constituting 47% of elongating and 36% of maturing cell total lipids by weight. PI also was a prevalent polar lipid in cotton fiber cells. Although values were variable when quantified by HPLC-ELSD, PI consistently was more abundant in maturing fibers, averaging 29% of total polar lipids, than in elongating fibers, where PI levels averaged 18% by weight of total polar lipids. PE and DGDG were present in similar proportions in elongating and maturing fibers from 11 to 14% by weight of total polar lipids. Relatively low levels of PA were observed, with only 2 and 4% (by weight) of total polar lipids in elongating and maturing fiber cells, respectively. The levels of acylated sterol glycoside (ASG) were higher in maturing fiber cells (7%) than in elongating fiber cells (2%), whereas similar levels of SG were observed at both developmental stages.

Recent advances MS methods have facilitated detailed class and molecular species analyses of plant glycerolipids (21). The classes and molecular species of polar lipids in elongating and maturing cotton fibers were determined by direct infusion ESI followed by tandem MS. This method does not incorporate HPLC or any other chromatographic separation. Instead, the species in each headgroup class are detected, as they produce a common headgroup fragment following collisionally activated dissociation. Classes are detected by sequential scans during continuous infusion, and quantification is in comparison with two internal standards of each headgroup class (21). The composition and relative proportions of the different glycerolipid classes were similar in elongating and maturing fiber cells (Fig. 4). The only major difference observed was in the relative amounts of lipid per dry weight (Fig. 4B), which was two times higher in elongating than in maturing fiber cells. This was consistent with results from total FA content measured above (Fig. 2) and is likely due to the increase in relative cellulose content at the later stages of fiber maturation. PC, PE, and PI were the predominant phospholipids in cotton fiber cells at both stages, and, consistent with data from HPLC-ELSD, the relative percentage of PI was somewhat higher in maturing than in elongating fiber cells (Fig. 4A).

PE constituted a considerably higher proportion of total polar lipid when measured by ESI-MS/MS (compared with HPLC-ELSD), about 30% of total polar lipid. It is possible that the lower levels of PE measured *via* HPLC-ELSD vs. *via* ESI-MS/MS may occur because PE (a basic lipid) is not accurately separated (chromatographed) by this system (even though the PE standard is used to determine the retention time and the PE

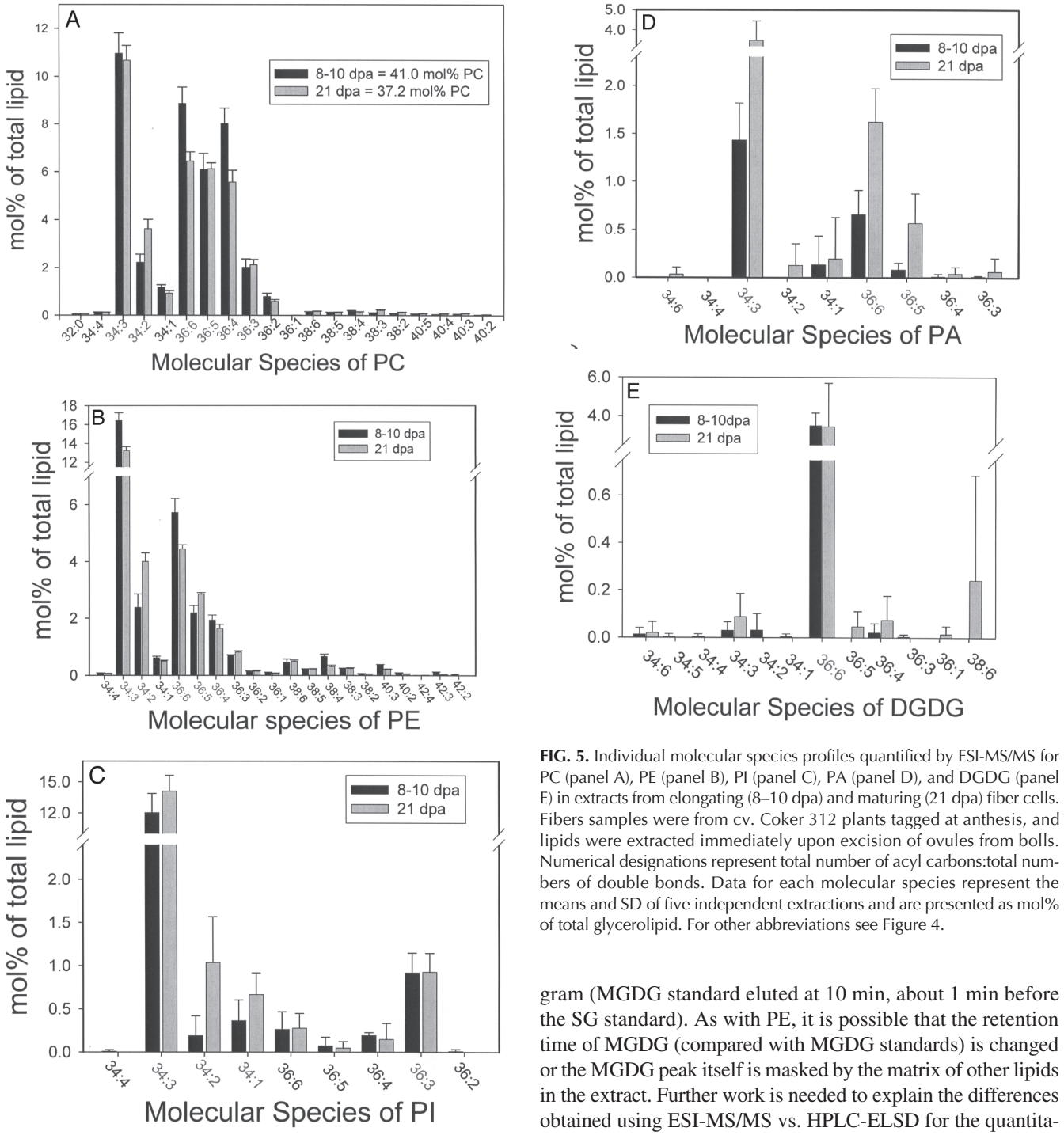

FIG. 5. Individual molecular species profiles quantified by ESI-MS/MS for PC (panel A), PE (panel B), PI (panel C), PA (panel D), and DGDG (panel E) in extracts from elongating (8–10 dpa) and maturing (21 dpa) fiber cells. Fibers samples were from cv. Coker 312 plants tagged at anthesis, and lipids were extracted immediately upon excision of ovules from bolls. Numerical designations represent total number of acyl carbons:total numbers of double bonds. Data for each molecular species represent the means and SD of five independent extractions and are presented as mol% of total glycerolipid. For other abbreviations see Figure 4.

standard is used to construct a calibration curve of PE mass vs. PE peak area. A possible explanation is that the retention time of PE (compared with PE standard, which elutes at 23 min) may be changed by the matrix of other lipids (or ions) in the extract, perhaps causing it to bind to another lipid and elute as part of a peak of another lipid class or to bind irreversibly to the column. It should similarly be noted that although MGDG is detected at significant levels *via* ESI-MS/MS (Fig. 4), no peak of MDGD was detected in the HPLC-ELSD chromatogram (MGDG standard eluted at 10 min, about 1 min before the SG standard). As with PE, it is possible that the retention time of MGDG (compared with MGDG standards) is changed or the MGDG peak itself is masked by the matrix of other lipids in the extract. Further work is needed to explain the differences obtained using ESI-MS/MS vs. HPLC-ELSD for the quantitative analysis of lipid classes. Although there were differences in the analytical results obtained by these two very different methods, overall trends and amounts of polar lipids quantified were similar.

The detailed molecular species profiles of the major glycerolipid classes were determined (Fig. 5). There were some similarities and differences in the profiles of glycerolipid molecular species, which are presented as the combined acyl composition. Major molecular species of PC and PE were quite similar (Fig. 5A, B), including 34:3 (16:0, 18:3), 34:2 (16:0,

18:2 or 16:1, 18:1), 34:1 (16:0, 18:1), 36:6 (18:3, 18:3), 36:5 (18:2, 18:3), 36:4 (18:1, 18:3 or 18:2, 18:2), and 36:3 (18:0, 18:3 or 18:1, 18:2). Relatively speaking, PE had more minor long-chain species (series of PE38, PE40, and PE42) than PC. PI molecular species profiles were much less complex than profiles of PC or PE (compare Fig. 5A–C), and were almost entirely dominated by PI 34:3 in fiber cells at both stages of development. The most abundant molecular species for PC, PE, PI, and PA was 34:3 (18:3,16:0). In addition, 36:6 (18:3,18:3) also was prevalent in PC, PE and PA, but not in PI (compare Fig. 5A–D). The composition and relative proportions of molecular species found in PA were similar to those found in PC and PE, suggesting that these PA species are indeed metabolites (precursors or products) of the two major phospholipids. The precise identity of the acyl groups is tentative in some of molecular species because multiple combinations of FA at the *sn*-1 and *sn*-2 positions of the glycerolipids can make up the reported total number of acyl carbons and double bonds. However, it should be noted that the prevalence of 18:3- and 16:0-containing molecular species is entirely reasonable, given total FA composition of the lipids in these cells (Fig. 1).

Relatively low levels of PS, PG, MGDG, and DGDG were present in cotton fiber lipid extracts analyzed by ESI and tandem MS (Fig. 4). Longer-chain FA were found to comprise the major molecular species in PS, with PS 40:3 being the most prevalent, and lower levels of PS 38:3 and PS 34:3 were detected (not shown). For PG, the most abundant molecular species were PG 32:0 (16:0, 16:0) and PG 32:1 (16:0,16:1); some PG 34:3 (18:3,16:0) was detected in fiber lipid samples as well (not shown). The major molecular species present in MGDG and DGDG were 36:6 (18:3, 18:3) (Fig. 5E).

Overall, there were subtle but significant differences in molecular species profiles between elongating and maturing fiber cells. The higher relative levels of 34:2 (16:0, 18:2) PC, PE, PI, and PA in the maturing as compared with the elongating cells are consistent with the higher levels of palmitic and linoleic acids in the maturing cells (Fig. 1). Higher levels of PE 36:5 (18:2, 18:3) may also reflect higher 18:2 levels, but higher levels in maturing fiber cells of PA 34:3, 36:5, and 36:6 than in elongating cells probably just reflect the higher total levels of PA in the maturing cells, as determined by ESI-MS/MS. On the other hand, PC 36:6, PC 36:4, PE 34:3, and PE 36:6 were higher on a mol% basis in elongating cells than in maturing fiber cells. It should be emphasized that polar lipid profiles were dramatically different when fiber samples were frozen prior to analysis; considerably higher amounts of PA (up to 40 mol%) were measured by both ESI-MS/MS and HPLC-ELSD in extracts from frozen fiber samples, and several pilot experiments determined that this could be attributed to the post-harvest hydrolytic activity of PLD. This was not observed with other cotton tissues (e.g., leaves) and indicates a considerable capacity for PLD hydrolysis in fiber cells. Caution should be exercised when handling and processing cotton fibers for polar lipid profiles.

Profile of EST in developing cotton fibers. Expressed genes in cotton fibers have been identified through several EST projects, and these publicly available sequences have been assembled into contigs by the efforts of researchers at The Institute for Genomic Research (http://www.tigr.org). These include EST from two libraries for cotton fibers at 7–10 dpa and 6 dpa, which is the stage of rapid elongation. We used these data to catalog EST for the major enzymes involved in lipid metabolism in elongating cotton fiber cells (Table 1). Approximately 39,000 EST have been derived from the 7–10 dpa library, as compared with only 8,000 from the 6 dpa library. Hence, the number of EST and TC sequences discussed here are mostly representative of the 7–10 dpa library, with certain noted exceptions. In some cases, the EST were already annotated, whereas in other instances homologs were tentatively identified by WU-BLAST with cDNA and/or protein sequences from other plants as queries. Many of these plant sequences were obtained from a lipid gene database for *A. thaliana* housed at http://www.plantbiology.msu.edu/lipids/genesurvey/index.htm (17) which also provides detailed reactions and metabolic pathway(s) for plant lipid metabolism. Hence, we also compared the total number of lipid enzyme EST from all tissues in *A. thaliana* to those in cotton fiber cells. The abundances of EST were used only as an indicator of the potential capacity for fiber cells to produce the different gene products and respective lipid metabolites.

EST for enzymes and proteins of *de novo* FA biosynthesis were prevalent in elongating cotton fiber cDNA libraries, including those encoding subunits of acetyl-CoA carboxylase, ACP, β-ketoacyl-ASP synthase I (KAS I), and stearoyl-ACP desaturase. Several EST were predicted to encode for different isoforms of ACP, including a fiber-specific ACP (13). No EST were annotated as encoding KAS II and KAS III enzymes, but KAS II shares high homology with KAS I, and some of the EST predicted to encode for KAS I could in fact encode for other enzymes. These results support metabolic data that cotton fiber cells at this stage of development have a high capacity for the *de novo* biosynthesis of FA and identify candidate cotton sequences that can be targeted for future functional studies.

The FA synthesized in the plastids can either be transferred from acyl-ACP into plastidial glycerolipids, or cleaved by a thioesterase to form free acyl chains that are exported to the ER for extraplastidial glycerolipid assembly (7). Three EST for FatA, the thioesterase that acts preferentially on unsaturated FA (22), and 11 EST for Fat B, which exhibits a preference for saturated acyl-ACP (23), were identified. Eleven EST were annotated as encoding for acyl-CoA synthetase, which esterifies the free acyl chains to CoA forming acyl-CoA (7), the precursors for ER glycerolipids.

Glycerolipid synthesis initiated by the transfer of acyl chains (from acyl-ACP in plastids or acyl-CoA in the ER) to glycerol-3-phosphate (G3P) forming lysophosphatidic acid (LPA) in a reaction is catalyzed by a G3P acyltransferase (7). Several EST were annotated as acyltransferases. Only a single EST from the 6 dpa library could be annotated as encoding an acyl-ACP acyltransferase. Nine EST, representing one TC, exhibited high identity with sequences of known acyl-CoA acyltransferases. In *A. thaliana*, only two EST for each of these enzymes have been

TABLE 1
Catalog of Expressed Sequence Tags (EST) Predicted to Encode for Enzymes and Other Proteins Involved in Lipid Metabolism in Two Cotton Fiber Libraries[a] (7–10 and 6 dpa)

Enzyme or protein		Cotton fiber			*Arabidopsis* (all tissues)	
		EST	EST	TC	EST	Genes
EC #	FA biosynthesis	7–10 dpa	6 dpa	7–10 dpa		
6.4.1.2	Acetyl-CoA carboxylase subunits	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	β-Ketoacyl-ACP reductase	3	2	1	35	5
4.2.1.*	β-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 thioester (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	LPA Acyltransferase-plastidial	13	0	1	7	1
2.3.1.51	LPA Acyltransferase-ER	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	DAG transferase	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 phosphotransferase)	2	0	1	3	1
2.7.8.5	ER PGP synthase	0	1	1	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	1	13	4
2.7.7.15	Phospholipid cytidyltransferase	4	0	1	8	2
2.7.8.2	Aminoalcoholphosphotransferase	2	0	1	11	1
1.14.99.*	ω-6 FAD ER (FAD2)	10	10	2	131	1
1.14.99.*	Plastidial FAD 6	1	0	1	9	1
1.14.99.*	ω-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	1	8	2
	Sphingolipid biosynthesis					
2.3.1.50	Serine palmitoyltransferase	4	2	2	13	3
1.1.1.102	3-Ketosphinganine reductase	29	2	2	18	2
	Acyl-CoA independent ceramide synthase	1	1	1	0	0
2.3.1.24	Sphinganine acyltransferase	0	0	0	0	0
	Δ 8 Sphingolipid desaturase	1	0	1	36	2
	FA elongation/wax synthesis					
6.2.1.3	Long-chain acyl-CoA synthetase	11	4	2	63	9
	β-Ketoacyl-CoA synthase	150	43	16	183	20
	β-Ketoacyl reductase	15	7	2	47	2
4.2.1.17	3-Hydroxyl-CoA 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	1	28	3
	CER3	0	5	1	7	1
	Lipid transfer protein precursor	151	254	4	362	14
	Lipid transfer protein 3 precursor	67	125	4	28	8
	Palmitoyl-protein thioester- like protein	2	2	1	44	7

(Continued)

TABLE 1 (*Continued*)

EC #	Enzyme or protein FA biosynthesis	Cotton fiber EST 7–10 dpa	 EST 6 dpa	 TC 7–10 dpa	*Arabidopsis* (all tissues) EST	 Genes
	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
	Δ 8 Δ 7 Isomerase	2	0	1	N/A	N/A
	Sterol-C4-α methyl oxidase	5	2	1	N/A	N/A
	UDP:glucose sterol glucosyl transferase	6	2	2	N/A	N/A
	Sterol methyltransferase	8	0	1	N/A	N/A
	Lipases					
3.1.4.4	Phospholipase D (α)	25	3	1	35	4
3.1.4.4	Phospholipase D (β1)	2	0	1	4	2
3.1.4.4	Phospholipase D (δ)	1	0	1	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
	FA amide hydrolase	16	7	5	5	2
	β-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-Hydroxybutyryl-CoA dehydrogenase	3	0	1	10	1
2.3.1.16	3-KetoacylCoA thiolase	5	0	1	65	3
	AcylCoA synthase	8	0	2	0	0

[a]The number of tentative consensus sequences (TCs) in the 7–10 dpa library is noted and may indicate the potential number of genes for each protein. The EST data were obtained from The Institute for Genomic Research (TIGR) through the web site at http://www.tigr.org. Comparisons were made to EST involved in lipid metabolism in *Arabidopsis thaliana*, using data compiled in the *Arabidopsis* lipid gene database housed at www.plantbiology.msu.edu/lipids/genesurvey/index.htm (17). Lipid metabolic genes for which no EST have been annotated in this database are marked as N/A. ACP, acyl carrier protein; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; ER, endoplasmic reticulum; PGP, phosphatidylglycerol phosphate; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

identified. Transfer of a second acyl chain to the *sn*-2 position of LPA forms PA, and several EST were identified for the plastidial and ER isoforms of LPA acyltransferase. The occurrence of these EST suggests that the synthesis of PA occurs in both the plastid and ER of fiber cells. Of interest, 66 cotton fiber EST appeared to encode acyltransferases of unidentified function.

PA serves as the precursor to most glycerolipids and is converted to DAG or cytidine diphosphate:diacylglycerol (CDP:DAG) for complex glycerolipid assembly (24). PA may be dephosphorylated by PA phosphatase, forming DAG, and a single EST predicted to encode this enzyme was present, whereas four EST (two TC) for DAG kinase (which catalyzes the reverse reaction) were identified. DAG also is used as a substrate in the synthesis of plastidial galactolipids, MGDG and DGDG. Only EST corresponding to DGDG synthase were identified. DAG may also be acylated by DAG acyltransferase to form TAG, but no EST were identified for this protein, nor were any EST identified as encoding a phospholipid diacylglycerol acytransferase, which catalyzes the transfer of an acyl group from a phospholipid to DAG forming a TAG and a lysophospholipid. These results might be expected since elongating fiber cells are not primary sites for TAG biosynthesis, but rather likely are more involved in the synthesis of membrane glycerolipids.

In the ER, DAG acts as a direct precursor for synthesis of PC and PE in the ER *via* the action of a phosphotransferase, displacing cytidine monophosphate (CMP) from the nucleotide-activated amino alcohols, CDP-choline or CDP ethanolamine. The amino alcohols are themselves produced in a series of reactions in which they are first phosphorylated, and then reacted with CTP to produce the CDP derivatives (24). Three cotton EST were identified as candidate choline/ethanolamine kinase homologs. Four EST were annotated as encoding the cytidyltransferase enzyme, while 2 EST were predicted to encode an aminoalcohol phosphotransferase. A single aminoalcohol phosphotransferase catalyzes the final step, producing PC or PE (25).

PA may be converted to CDP:DAG by action of CDP:DAG synthase, but surprisingly no EST encoding for this enzyme were identified. CDP:DAG is the precursor for PG, PI, and PS (26). Phosphatidylglycerol phosphate (PGP) synthase uses CDP:DAG and G3P to form PGP, which is the precursor to PG, and 2 EST were predicted to encode for the plastidial isoform of PGP synthase, while a single EST in the 6 dpa library was annotated as encoding for the ER isoform. However, no EST were identified as encoding for PGP phosphatase, which produces PG from PGP. Additionally, no EST were predicted to encode for PI synthase or PS synthase, which both use CDP:DAG as a precursor.

Desaturation of glycerolipid acyl chains occurs in both the plastid and ER and is catalyzed by different proteins in these

organelles. Ten EST predicted to encode for the ER δ-12 desaturase [fatty acid desaturase 2 (FAD2)] were identified, while only a single EST was annotated as encoding the plastidial isoform, FAD6. Five EST were identified as an ER Δ-15 desaturase (FAD3), while eight EST were predicted to encode the plastidial isoforms FAD7/FAD8.

The large number of EST predicted to code for enzymes involved in FA elongation and wax biosynthesis suggests that elongating fiber cells are actively engaged in these processes. The FA elongase system, which adds two carbons per cycle to C_{18}-acyl-CoA, consists of four components; β-ketoacyl-CoA synthase, β-ketoacyl -CoA reductase, β-hydroxylacyl-CoA dehydratase, and enoyl-CoA reductase (23). A large number of EST (150) were identified as encoding for β-ketoacyl-CoA synthase, while15 EST were annotated as encoding for the reductase, but no EST were identified for the latter two components of the pathway. A surprisingly high number of EST were identified for lipid transfer protein precursors, but this is a feature of many cDNA libraries. A total of 218 EST in the 7–10 dpa library and 379 EST in the 6 dpa library were annotated as encoding for this type of protein. Lipid transfer proteins are thought to have a role in cutin/wax deposition (27), and fiber-specific transcripts of this enzyme that were highly expressed during the stage of rapid elongation have been identified in cotton (14,16).

Sphingolipids are constituents of membranes (particularly, plasma membranes), and several EST for enzymes involved in their biosynthesis were identified in the cotton fiber databases. Four EST were annotated as encoding a serine palmitoyltransferase, which catalyzes the condensation reaction between serine and palmitoyl-CoA forming 3-ketosphinganine in the first step of the pathway (28). Several EST were identified for 3-ketosphinganine reductase, which forms sphinganine. Ceramide synthesis is thought to occur either by acylation of sphinganine with a free FFA or an acyl-Co A donor (28). Ceramide synthase catalyzes the former reaction, and a single EST was identified for this enzyme, while no EST were annotated as encoding for sphinganine acyltransferase, which catalyzes the latter reaction. A single EST predicted to encode a sphingolipid desaturase was identified.

Sterols are minor components of cotton fiber (29), and several EST encoding enzymes involved in sterol biosynthesis were identified in the cotton fiber databases. These include 21 EST for HMG-CoA reductase, a major precursor in sterol biosynthesis, 14 EST for 24 sterol methytransferase, 2 EST for sterol C_{14} reductase, 2 EST for the Δ 8, Δ 7 isomerase, 5 EST for sterol 4-α methyl oxidase, 6 EST for UDP-glucose: sterol glucosyltransferase, and 8 EST annotated as a *S*-adenosyl-methionine-sterol methyltransferase.

Plant lipids undergo membrane remodeling during growth and development, as well as in response to various environmental conditions, and lipases are involved in these processes (30). PLD is known to hydrolyze phospholids producing PA and a headgroup, and multiple isoforms of this enzyme have been identified in plants (31). In the cotton fiber libraries, the most abundant PLD EST were those for PLDβ, for which 25 EST were identified, while only 2 EST were identified as encoding for PLDβ and a single EST was identified for the PLD δ isoform. Eighty-four EST were grouped as homologs of lysophospholipases. Two EST were specifically annotated as encoding phospholipase A1, which cleaves phospholipids at the *sn*-1 position, while 7 EST were predicted to encode phospholipase A2, which cleaves phospholipids at the *sn*-2 position. Additionally, 12 EST were annotated as encoding PI-specific phospholipase C, suggesting that PI-mediated signaling may be a feature of cotton fiber cell elongation. Orthologs of the recently discovered plant FA amide hydrolase (32) also were expressed in developing cotton fibers.

FA are turned over in plant cells by the peroxisomal β-oxidation pathway (33). Several EST for each of the enzymes involved in this process were present in the cotton fiber databases. These included 8 EST for acyl-CoA synthase, 4 EST for acyl-CoA oxidase, 7 EST for enoyl-CoA hydratase, 3 EST for 3-hydroxybutyryl-CoA dehydrogenase, and 5 EST for 3-ketoacyl-CoA thiolase. The expression of these genes in elongating cotton fiber cells suggests that FA degradation occurs even at the early stages of fiber development.

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 plant cell expansion (6). The rapid elongation of these cells requires substantial lipid synthesis to support the developing organelle and membranes, and this was supported by estimates of acyl lipid content at several stages of fiber development (Fig. 2). The presence of several EST for enzymes involved in FA biosynthesis indicated that this pathway is quite active at the early stage of fiber development (Table 1). A large number of EST were predicted to encode for subunits of acetyl-CoA carboxylase, which is considered to be the major regulatory enzyme in the pathway (34). Several EST were identified for ACP, which carry the FA through the assembly process, as well as for KAS I, which participates in fatty acyl chain elongation. 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 hypothesize that some of the EST annotated as encoding for KAS I may in fact be KAS II EST, owing to the high sequence homology. Several EST for stearoyl-ACP desaturase, which introduces a double bond in stearate (18:0) to form 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 FA are then incorporated into complex lipids during plastidial and extraplastidial glycerolipid synthesis and modification. It should be emphasized that additional quantitative proteomics data (or western blotting) will be required to provide information about actual abundance of proteins in these pathways. Nonetheless, lipid

and EST profiles do confirm the metabolic capacity of cotton fiber cells for these pathways.

The molecular species composition of glycerolipids can be used to gain information as to how these lipids might be synthesized in cells. For example, glycerolipids are usually synthesized in plants from a PA precursor *via* two pathways, the prokaryotic and the eukaryotic pathway (35). In the prokaryotic pathway, 16-carbon FA are found predominantly in the *sn*-2 position. In the eukaryotic pathway, 16-carbon FA, when present, are found at the *sn*-1 position, and 18-carbon FA can be esterified to either position (36). Although acyl positions have only been tentatively assigned to the species identified by ESI-MS/MS, some conclusions can nonetheless be drawn from results such as those presented in Figure 5. DGDG (and MGDG) contained 36:6 (18:3,18:3) as the most abundant molecular species (Fig. 5E); this points to the DAG moieties of these lipids being synthesized *via* the eukaryotic pathway, since this is the only mechanism by which 18-carbon acyl chains are placed at both *sn*-1 and *sn*-2 positions. On the other hand, most of the PG is likely to be synthesized by the prokaryotic pathway, since the major molecular species of PG were 32:0 (16:0, 16:0) and 32:1 (16:0,16:1) (not shown).

In this same context, it is possible to deduce the scheme by which the major phospholipid species are synthesized in cotton fibers, and the EST occurrence supports the metabolic compartmentation scheme for PC (and PE) biosynthesis shown in Figure 6. As in most plant cell types, 16:0 and 18:1 FA are the major FA synthesized in and exported from plastids to the ER for complex lipid formation. These FA are incorporated *via* CoA esters into the *sn*-1 and -2 position of G3P by G3P acyltransferase and LPA acyltransferase, respectively, to form PA. Although not shown, 18:1 (or 18:0) could be incorporated into either acyl position of PA. PA is dephosphorylated to yield DAG, which is used for the synthesis of PC (and PE) *via* the membrane-bound aminoalcohol phosphotransferase enzyme with the appropriate CDP donor. The 18:1 acyl chains are modified by the Δ-12 (FAD2) and the Δ-15 (FAD3) desaturases to yield 18:2 and 18:3 FA.

The principal molecular species of PC (16:0, 18:3) and PE (16:0, 18:3) together were about 25 mol% of the total polar lipid fraction (Figs. 5A and 5B). These two lipids are likely to be the principal phospholipid components of both vacuole and plasma membranes of expanding cotton fiber cells, and they are most likely synthesized by cooperation between the plastidial and ER compartments *via* the scheme shown in Figure 6. Additional molecular species for these two lipid classes wherein 18-carbon FA are at both positions (36:6, 36:5, 36:4, 36:3) together were the majority of the remaining molecular species of both PC and PE, and these metabolites are likely to be synthesized by the same machinery. EST for all of these enzymatic steps were identified, so the molecular informatics and metabolite profiles are consistent for the major phospholipid biosynthetic pathways in elongating cotton fiber cells.

The major molecular species of PI was 34:3, and the acylglycerol backbone could be synthesized using the same machinery for the major PC species described above. Less clear is how the headgroup of PI is incorporated into this glycerolipid class. No EST were predicted to encode for PI synthase (or PS synthase), which uses CDP:DAG as a precursor. The DAG base could be derived from the PC or PE pathway, but the headgroup metabolism is unclear since no CDP:DAG transferase EST were identified. This is somewhat surprising given the particular abundance of PI in fiber cells, and may indicate that these enzymes have rare or substantially diverged messages not yet identified in these libraries, or that PI, PS, and perhaps even PG are produced *via* alternative pathways in cotton fiber cells.

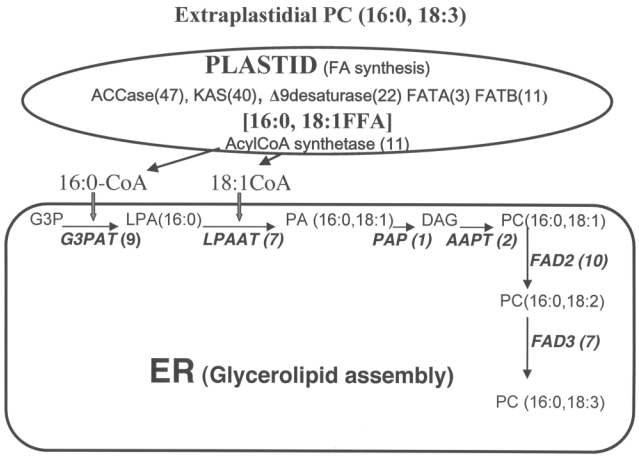

FIG. 6. Summary scheme for the cellular compartmentation and proposed pathway leading to the synthesis of PC 34:3 (16:0, 18:3), a major glycerolipid molecular species in both elongating and maturing fiber cells. The diagram is based on both metabolite profiles (Figs. 1–5) and expressed sequence tag (EST) occurrences (numbers in parentheses) for enzymes in these pathways (Table 1), and is consistent with the synthetic pathways described for extraplastidial PC in other types of plant cells (35). The FA are assembled *de novo* in the plastids of fiber cells and exported to the endoplasmic reticulum (ER) for glycerolipid assembly and subsequent modification (e.g., introduction of additional double bonds). The acylglycerol base for other phospholipids, such as PE and PI, can be synthesized using the same machinery. ACCase, acetyl-CoA carboxylase; KAS, ketoacyl ACP synthase; FatA, FA thioesterase A; FatB, FA thioesterase B; G3P, glycerol-3-phosphate; G3PAT, G3P acyltransferase; LPA, lysophosphatidic acid; LPAAT, LPA acyltransferase; PAP, PA phosphatase; AAPT, aminoalcohol phosphotransferase; FAD, FA desaturase. Numerical designations for FA represent total number of acyl carbons:total numbers of double bonds.

From the lipid metabolite data, it is possible to make some predictions about how lipid metabolism may be directed in fiber cells as they shift from the rapid elongation phase to the maturation phase. For example, there was a significant reduction in PE 34:3 and PE 34:6, whereas there was an increase in PE 34:2 and a slight increase in PE 34:5. A similar increase was noted in PC 34:2 with a decrease in PC 36:6. These results suggest a reduction in FAD3 activity during the maturation phase and could impart a subtle change in membrane fluidity to fiber cells.

By combining lipid metabolite data with EST profiles it may be possible to identify lipid metabolic pathways important in fiber cells that have not been appreciated previously. For example, saturated FA such as 16:0 and 18:0 are important for

membrane stability, but they also may be elongated to very long-chain FA that serve as precursors for the biosynthesis of surface components such as waxes (27,37). A major amount of 16:0 FA was detected in fibers at all stages of elongation and maturation (Fig. 1). Moreover, an abundance of EST for FA elongation and wax biosynthesis enzymes indicated that elongating fibers may be actively involved in these processes and would certainly require the necessary acyl chain precursors.

Lipid profiling and EST data suggest that sterol metabolism may be important to cotton fiber development, as has been suggested by others (11). ASG levels were higher in maturing fiber cells than in elongating fiber cells (Fig. 3), and ASG is mostly associated with plasma membranes (10,38,39). Others have implicated SG in the biosynthesis of cellulose (11). EST were identified for several sterol/SG enzymes in the elongating fibers, and these represent excellent targets to test the importance of sterol-containing lipids in the shift of fiber cells from the synthesis of primary to secondary cell walls.

Likewise, lipid profiling and EST data may suggest that PLD plays a role in cotton fiber development. PLD EST were quite prevalent in elongating fiber libraries, and the rapid, albeit artifactual, production of PA in frozen fiber cell extracts suggests that there is a substantial capacity for PLD-mediated phospholipid metabolism in fiber cells. It is possible that this signifies that PLD has an important function in the normal development of cotton fiber cells. PLD are thought to interact with cytoskeletal filaments and to influence cell expansion (40–42), and their role in membrane trafficking in mammalian systems is well documented (43). Perhaps the results gleaned from these lipidomics approaches will prompt additional molecular and biochemical experiments to characterize the impact of surface lipids, sterol metabolism, lipid hydrolases, and the like on the development of cotton fibers.

To our knowledge these studies represent the first attempt to reconcile detailed lipid metabolite profiles and EST profiles from DNA databases for this unusual and economically important cell type. It is anticipated that these results will provide the basis for continued efforts to understand the coordinated regulation of cotton fiber development at the molecular, biochemical and physiological levels, and that this approach, originally applied to *Arabidopsis* seeds (17), will encourage additional efforts to integrate metabolic and genomics information to improve our understanding of the importance of lipid metabolism to plant cell growth and development.

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REFERENCES

1. Lang, A.G. (1938) Origin of Lint and Fuzz Hairs in Cotton, *J. Agric. Res. 56*, 507–521.
2. Basra, A.S., and Malik, P. (1984) Development of Cotton Fiber, *Int. Rev. Cytol. 89*, 65–109.
3. Ramsey, J.C., and Berlin, J.D. (1976) Ultrastructural Aspects of Early Stages in Cotton Fiber Elongation, *Am. J. Bot. 63*, 868–876.
4. Stewart, J.D. (1975) Fiber Initiation on the Cotton Ovule (*Gossypium hirsutum*), *Am. J. Bot. 62*, 723–730.
5. Schubert, A.M., Benedict, J.D., Berlin, J.D., and Kohel, R.J. (1973) Kinetics of Cell Elongation and Secondary Cell Wall Thickening, *Crop Sci. 13*, 704–709.
6. Kim, H.J., and Triplett, B.A. (2001) Cotton Fiber Growth *in planta* and *in vitro*. Models for Plant Cell Elongation and Cell Wall Biogenesis, *Plant Physiol. 127*, 1361–1366.
7. Somerville, C., Browse, J., Jaworski, J.G., and Ohlrogge, J.B. (2000) Lipids, in *Biochemistry & Molecular Biology of Plants* (Buchanan, B., Gruissem, W., and Jones, R., eds.), pp. 456–527, American Society of Plant Physiologists, Rockville, MD.
8. Welti, R., Li, M., Li, W., Sang, Y., Biesiada, H., Zhou, H.-E., Rajashekar, C., Williams, T., and Wang, X. (2002) Profiling Membrane Lipids in Plant Stress Response, *J. Biol. Chem. 277*, 31994–32002.
9. Christie, W.W. (1992) Detectors for High-Performance Liquid Chromatography of Lipids with Special Reference to Evaporative Light-Scattering Detection, in *Advances in Lipid Methodology—One*, pp. 239–271, The Oily Press, Ayr, Scotland.
10. Moreau, R.A., Powell, M.J., and Singh, V. (2003) Pressurized Liquid Extraction of Polar and Nonpolar Lipids in Corn and Oats with Hexane, Methylene Chloride, Isopropanol, and Ethanol, *J. Am. Oil Chem. Soc. 80*, 1063–1067.
11. Peng, L., Kawagoe, Y., Hogan, P., and Delmer, D. (2002) Sitosterol-β-glucoside as a Primer for Cellulose Synthesis in Plants, *Science 295*, 147–149.
12. Ji, S.J., Lu, Y.C., Feng, J.X., Wei, G., Li, J., Shi, Y.H., Fu, Q., Liu, D., Luo, J.C., and Zhu, Y.X. (2003) Isolation and Analyses of Genes Preferentially Expressed During Early Cotton Fiber Development by Subtractive PCR and cDNA Array, *Nucleic Acids Res. 31*, 2534–2543.
13. Song, P., and Allen, R. (1997) Identification of a Cotton Fiber Specific Acyl Carrier Protein cDNA by Differential Display, *Biochim. Biophys. Acta 1351*, 305–312.
14. Orford, S.J., and Timmis, J.N. (2000) Expression of a Lipid Transfer Protein Gene Family During Cotton Fiber Development, *Biochim. Biophys. Acta 1483*, 275–284.
15. Orford, S.J., and Timmis, J.N. (1997) Abundant mRNAs Specific to the Developing Cotton Fibre, *Theor. Appl. Genet. 94*, 909–918.
16. Ma, D.P., Tan, H., Si, Y., Creech, R.G., and Jenkins, J.N. (1995) Differential Expression of a Lipid Transfer Protein Gene in Cotton Fiber, *Biochim. Biophys. Acta 1257*, 81–84.
17. Beisson, F., Koo, A.J., Ruuska, S., Schwender, J., Pollard, M., Thelen, J.J., Paddock, T., Salas, J.J., Savage, L., Milcamps, A., *et al*. (2003) Arabidopsis Genes Involved in Acyl Lipid Metabolism. A 2003 Census of the Candidates, a Study of the Distribution of Expressed Sequence Tags in Organs, and a Web-Based Database, *Plant Physiol. 132*, 681–697.
18. Chapman, K.D., and Moore, T.S., Jr. (1993) *N*-Acylphosphatidylethanolamine Synthesis in Plants: Occurrence, Molecular Composition, and Phospholipid Origin, *Arch. Biochem. Biophys. 301*, 21–33.
19. Brugger, B., Erben, G., Sandhoff, R., Wieland, F.T., and Lehmann, W.D. (1997) Quantitative Analysis of Biological Membrane Lipids at the Low Picomole Level by Nano-Electrospray Ionization Tandem Mass Spectrometry, *Proc. Natl. Acad. Sci. USA 94*, 2339–2344.

20. Kim, Y.H., Choi, J.S., Yoo, J.S., Park, Y.M., and Kim, M.S. (1999) Structural Identification of Glycerolipid Molecular Species Isolated from Cyanobacterium *Synechocystis* sp. PCC 6803 Using Fast Atom Bombardment Tandem Mass Spectrometry, *Anal. Biochem. 267*, 260–270.
21. Welti, R., and Wang, X. (2004) Lipid Species Profiling: A High Throughput Approach to Identify Lipid Compositional Changes and Determine the Function of Genes Involved in Lipid Metabolism and Signaling, *Curr. Opin. Plant Biol. 7*, 337–344.
22. Salas, J.J., and Ohlrogge, J.B. (2002) Characterization of Substrate Specificity of Plant FatA and FatB Acyl-ACP Thioesterases, *Arch. Biochem. Biophys. 403*, 25–34.
23. Huynh, T.T., Pirtle, P.R., and Chapman, K.D. (2002) Expression of a *Gossypium hirsutum* cDNA Encoding a FatB Palmitoyl-Acyl Carrier Protein Thioesterase in *Eschericia coli, Plant Physiol. Biochem.* 40, 1–10.
24. Kinney, A. (1993) Phospholipid Head Groups, in *Lipid Metabolism in Plants* (Moore, T.S., ed.), pp. 259–284, CRC Press, Boca Raton, FL.
25. Goode, J.H., and Dewey, R.E. (1999) Characterization of Aminoalcoholphosphotransferases from *Arabidopsis thaliana* and Soybean, *Plant Physiol. Biochem. 37*, 445–457.
26. Joyard, J. (1993) Origin and Synthesis of Galactolipid and Sulfolipid Head Groups, in *Lipid Metabolism in Plants* (Moore, T.S., ed.), pp. 231–258, CRC Press, Boca Raton, FL.
27. Kunst, L., and Samuels, A.L. (2003) Biosynthesis and Secretion of Plant Cuticular Wax, *Prog. Lipid Res. 42*, 51–80.
28. Lynch, D.V. (1993) Sphingolipids, in *Lipid Metabolism in Plants* (Moore, T.S., ed.), pp. 285–308, CRC Press, Boca Raton, FL.
29. Amin, S.A., and Truter, E.V. (1972) Cotton Lipids: A Preliminary Survey, *J. Sci. Food Agric. 23*, 39–44.
30. Chapman, K.D. (1998) Phospholipase Activity During Plant Growth and Development and in Response to Environmental Stress, *Trends Plant Sci. 3*, 419–426.
31. Wang, X. (2000) Multiple Isoforms of Phospholipase D in Plants: The Gene Family, Catalytic and Regulatory Properties and Cellular Functions, *Prog. Lipid Res. 39*, 109–149.
32. Chapman, K.D. (2004) Occurrence, Metabolism, and Prospective Functions of *N*-Acylethanolamines in Plants, *Prog. Lipid Res. 43*, 302–327.
33. Graham, I.A., and Eastmond, P.J. (2002) Pathways of Straight and Branched Chain Fatty Acid Catabolism in Higher Plants, *Prog. Lipid Res. 41*, 156–181.
34. Ohlrogge, J.B., Jaworski, J.G., and Post-Beittenmiller, D. (1993) *De Novo Fatty Acid Biosynthesis*, CRC Press, Boca Raton, FL.
35. Ohlrogge, J.B., and Browse, J. (1995) Lipid Biosynthesis, *Plant Cell 7*, 957–970.
36. Frentzen, M. (1993) Acyltransferases and Triacylglycerols, in *Lipid Metabolism in Plants* (Moore, T.S., ed.), pp. 195–230, CRC Press, Boca Raton, FL.
37. Post-Beittenmiller, D. (1996) Biochemistry and Molecular Biology of Wax Production in Plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol. 47*, 405–430.
38. Moreau, R.A., Powell, M.J., Whitaker, B.D., Bailey, B.A., and Anderson, J.D. (1994) Xylanase Treatment of Plant Cells Induces Glycosylation and Fatty Acylation of Phytosterols, *Physiol. Plant. 91*, 575–580.
39. Moreau, R., Whitaker, B.D., and Hicks, K.B. (2002) Phytosterols, Phytostanols, and Their Conjugates in Foods: Structural Diversity, Quantitative Analysis, and Health-Promoting Uses, *Prog. Lipid Res. 41*, 457–500.
40. Dhonukshe, P., Laxalt, A.M., Goedhart, J., Gadella, T.W., and Munnik, T. (2003) Phospholipase D Activation Correlates with Microtubule Reorganization in Living Plant Cells, *Plant Cell 15*, 2666–2679.
41. Gardiner, J., Collings, D.A., Harper, J.D., and Marc, J. (2003) The Effects of the Phospholipase D-Antagonist 1-Butanol on Seedling Development and Microtubule Organisation in *Arabidopsis*, *Plant Cell Physiol. 44*, 687–696.
42. Gardiner, J.C., Harper, J.D., Weerakoon, N.D., Collings, D.A., Ritchie, S., Gilroy, S., Cyr, R.J., and Marc, J. (2001) A 90-Kd Phospholipase D from Tobacco Binds to Microtubules and the Plasma Membrane, *Plant Cell 13*, 2143–2158.
43. Cockcroft, S. (2001) Signalling Roles of Mammalian Phospholipase D1 and D2, *Cell. Mol. Life Sci. 58*, 1674–1687.

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