Chapter 4

QUANTITATIVE AND SPATIAL ANALYSIS OF LIPID METABOLITES IN SEEDS OF DIVERSE *GOSSYPIUM* GENOTYPES

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Abbreviations- MALDI: matrix assisted laser desorption/ionization, MS: mass spectrometry, ESI: electron sprayionization, TD-NMR: time domain ^{1}H nuclear magnetic resonance, PC: phosphatidylcholine, TAG: triacylglycerol, TLE: total lipid extract, P: palmitic (16:0), O: oleic (18:1), L: linoleic (18:2) numerical designation of lipids indicates number of carbons in acyl chains: number of double bonds.

INTRODUCTION

Although cotton is farmed for its production of spinnable fibers, the residual seed after ginning is a plentiful source of vegetable oil. In fact, the yield of seed on a per acre basis is about 1.6 times that of the harvested fiber, and in 2012 this amounted to 5.37 million tons of cottonseed produced in the United States (USDA-Oil Crops Outlook). Currently, less than half of the seed produced in the United States is crushed and processed into refined vegetable oil, and with world demand for vegetable oils on the rise, this may represent a place for farmers to recognize additional value from their overall crop. Oilseeds and their refined vegetable oils vary in price based on their compositional formulations and end-use markets. Due to its oxidative stability and flavor enhancing properties, cottonseed oil enjoys a reputation as an excellent frying oil, but with changes to its fatty acid composition, cottonseed oil might enter other markets (Lui *et al.*, 2009; 2012). As with all natural products, the compositions of extracted products may vary from season to season, with environmental and genetic factors contributing to both desirable and undesirable components. A more complete understanding of the many components in refined cottonseed oils and the factors which influence their formation within the embryo may help to develop new varieties with consistent and highly desirable vegetable oil compositions. Detailed chemical analysis of seed oils (including minor components) within the context of different genotypes or environmental conditions could help to provide breeders with rich resources to enhance the overall value of the cotton crop. Moreover, the detailed analysis of lipid metabolites within embryos may offer insights into pathways and postharvest processes that influence seed viability and seedling vigor.

ANALYSIS OF COTTONSEED CONSTITUENTS

Oil and protein reserves in cotton seeds have been traditionally quantified using destructive and time-intensive chemical extractions (AOCS, 2009a; 2009b; 2009c). Advances in non-invasive methodologies, including time domain ^{1}H- nuclear magnetic resonance (TD-^{1}H NMR), have made it possible to quantify oil and protein content in seed samples without destroying viability (Horn *et al.*, 2011a). Cottonseed from current commercial cultivars typically averages about 20% oil and 25% protein by weight (Jones and King, 1996). A recent survey of oil and protein content within the genetically diverse U.S. National Cotton Germplasm Collection (Horn, Hinze, Percy and Chapman, unpublished observations) suggested that there was considerable variation in seed reserve composition within the *Gossypium* background. Here, eight accessions were identified with extreme levels of oil and/or protein reserves (Table 1) and were selected for further detailed lipid analysis. For example, oil content ranged from 8.2% by weight in *G. stocksii* to 25.5% by weight in *G. barbadense* (cv. Pima-S6), whereas protein content ranged from 11.4% in *G. thurberi* to over 30% by weight in a couple of *G. hirsutum* accessions (Table 1). Seed sizes ranged dramatically as well in these different accessions (Figure 1), and generally the larger seeds had a larger percentage of oil, but this positive trend was not observed for protein (i.e., there were large seeds with low protein like Pima-S6 and small seeds with higher protein like *G. stocksii* accession E01-3). Cultivated varieties examined included *G. barbadense*, cv Pima- S6, *G. hirsutum*, accession SA-1254 and *G. hirsutum* cv Coker 312 while others mostly were wild accessions (Table 1).

Table 1. Comparison of seed size, % oil (by weight), and % protein (by weight) for selected *Gossypium* accessions representing a broad range of oil and protein content across diverse genomes in the U.S. National Cotton Germplasm Collection. Oil and protein content was determined by TD-NMR in triplicate batches of seeds at approximately 1g each. Values are means and standard deviations.

Sample ID	Species	Genome	Status/Origin	Seed Size (mg/seed)	Oil (%)	Protein (%)
B01-1	G. anomalum	B1	Wild/Africa	24.4	12.8 ± 0.3	20.2 ± 0.3
D01-10	G. thurberi	D1	Wild/North America	21.0	16.6 ± 0.3	11.4 ± 0.3
E01-3	G. stocksii	E1	Wild/Arabia	24.1	8.2 ± 0.3	23.8 ± 0.3
PIMA-S6	G. barbadense	(AD)2	Cultivated/South America	119.2	25.5 ±0.3	16.3 ± 0.4
SA-1254	G. hirsutum	(AD)1	Cultivated/Central, North America	98.7	20.0 ± 0.4	37.4 ± 2.1
TX-2236	G. hirsutum	(AD)1	Wild/Central, North America	59.5	14.4 ± 0.1	31.0 ± 0.4
TX-2500	G. hirsutum	(AD)1	Wild/Central, North America	90.2	24.8 ± 0.1	23.0 ± 0.5
Coker 312	G. hirsutum	(AD)1	Cultivated/Central, North America	83.9	20.6 ± 0.3	24.0 ± 1.5

While TD-NMR provides accurate quantitative information on total seed lipid content, it does not provide detailed lipid composition information. Instead, direct-infusion, "shotgun" lipidomics analysis of total lipid extracts (TLE) of seeds by electrospray ionization mass spectrometry (ESI-MS) has made it possible to quantify individual lipid molecular species in cottonseeds in a relatively high throughput manner (Horn *et al.*, 2011b; 2012). Generally this "shotgun" lipidomics methodology complements well other analytical approaches historically used for the analysis of cottonseed lipids, such as gas chromatography (GC) (Metcalfe *et al.*, 1966; Bland *et al.*, 1991; Yunusova *et al.*, 1991; Dowd *et al.*, 2010) and high performance liquid chromatography (HPLC) (Bland *et al.*, 1991; Lísa and Holcapek, 2008) which often can be coupled to detection by mass spectrometry (e.g., GC-MS and LC-MS). A major advantage of the direct-infusion approach compared to the chromatographic approaches is a reduced time of sample handling/analysis without compromising the detailed, comprehensive and sensitive quantitative information for lipid composition (Horn and Chapman, 2012).

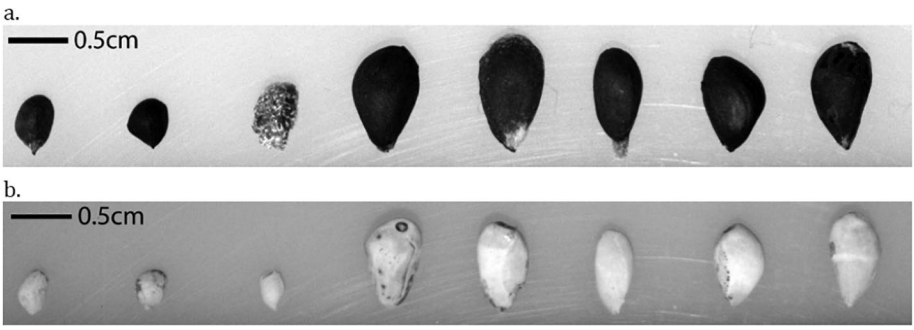


Figure 1. Representative mature seeds from genetically diverse Gossypium species with (a) and without seed coat (b). Sample IDs from left to right: B01-1, D01-10, E01-3, PIMA-S6, SA-1254, TX-2236, TX-2500 and Coker-312. See Table 1 for species, origins, and characteristics.

Cottonseed oil is mostly comprised of triacylglycerols (TAGs, ~98 % of the total lipid), and the molecular species profiles (differing in acyl chain composition) together make up the overall fatty acid composition of the oil. The major fatty acid species represented in cottonseed TAGs are palmitic (16:0), oleic (18:1), and linoleic (18:2) acids. TAGs from seeds of these selected accessions were quantified individually by ESI-MS, and there were clear differences in the fatty acid composition in TAGs among the seven genotypes examined (Figure 2A). One obvious difference was in the mol% of TAG 52:4, which contains acyl chains of 16:0/18:2/18:2. This molecular species made up a larger percentage of the total TAGs in several diploid exotic species and less in several (but not all) of the tetraploid genotypes. However, since few accessions were profiled here, it is also possible that similar variation would be seen across all genotypes. In any case, such marked variation in TAG composition suggests considerable genetic diversity in *Gossypium* to realize breeder-directed changes in seed oil composition.

Like in other oilseeds, TAGs are synthesized in cotton embryos from the metabolic precursor, phosphatidylcholines (PCs), by a relatively complex, and incompletely understood set of reactions (Chapman and Ohlrogge, 2012). The acyl chains that are assembled into TAGs are first incorporated and modified on PCs before being transferred to the glycerol backbone of TAGs by either acyl CoA-dependent or acyl CoA-independent pathways. Regardless of the route of entry, analysis of the acyl chains on PCs reflects a major precursor pool of metabolites for TAG synthesis, especially when analyzed in the same samples as for TAGs. As might be expected, the PC molecular species profiles were different among these diverse genotypes in a manner consistent with their corresponding TAGs (Figure 2B). For example, the accession E01-3, *G. stocksii*, was notably high in TAG 54:6, which is a TAG species with 18:2 at each position. Similarly, E01-3 was the genotype with the highest proportion of PC 36:4, which is a PC with 18:2 at each position, consistent with a precursor-product relationship between these PC and TAG molecular species.

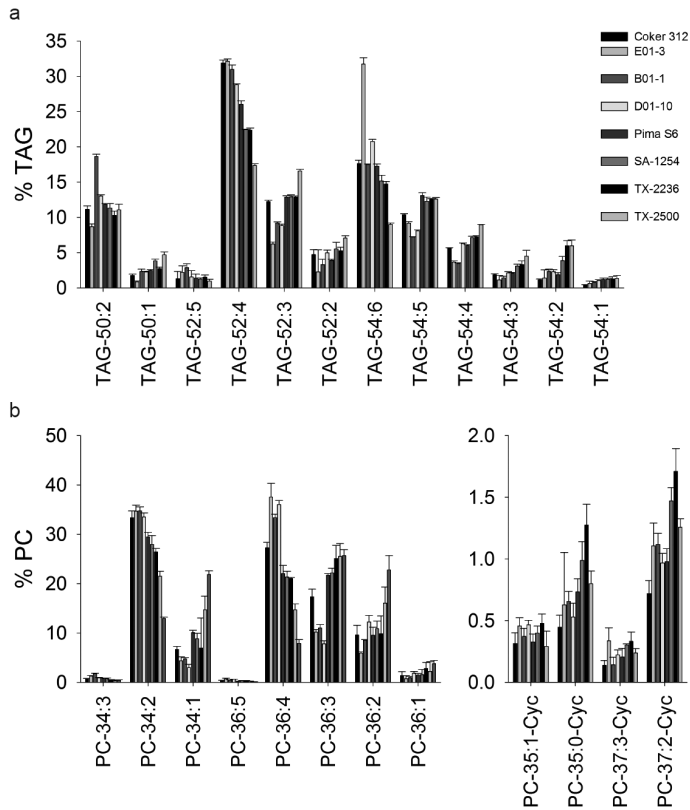


Figure 2. Direct infusion, "shotgun" lipidomics analysis of total lipid extracts (TLE) from seeds of diverse *Gossypium* species (see Table 1). Relative quantification of major TAG (a) and PC (b) molecular species are presented on a mol% basis within the class and were measured in triplicate with 5 seeds each in each replicate (bars represent standard deviation). Tandem MS scanning mode confirmed acyl chain composition (not shown here).

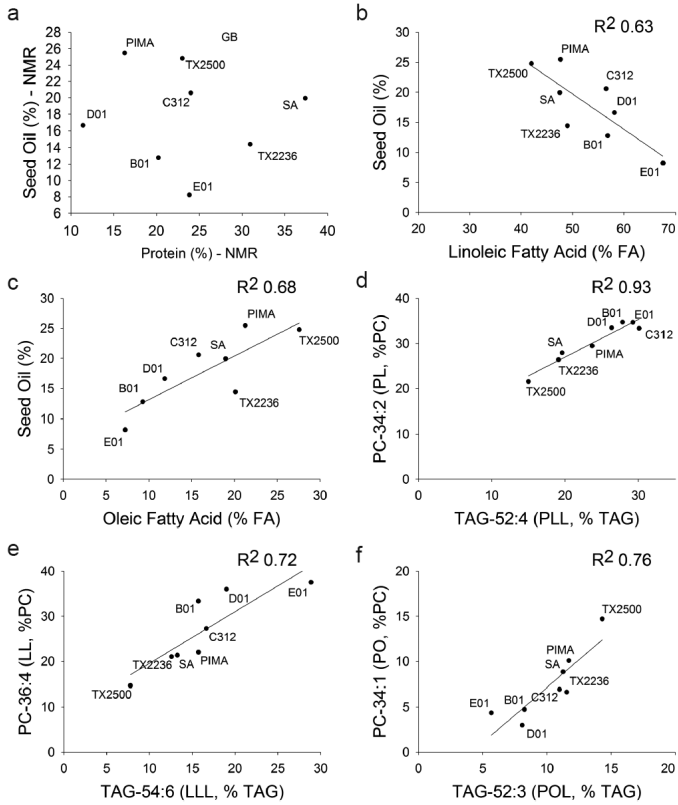


Figure 3. Lipids in seeds of diverse *Gossypium* species (see Table 1 for list). (a) Comparison of the seed oil and protein % by TD-NMR demonstrating variability in extremes chosen for further analysis. (b) Relationship of seed oil content and linoleic acid %. (c) Relationship of seed oil content and oleic acid %. (d) Relationship of mol% PC 34:2 (PL) and mol% TAG-52:4 (PLL). (e) Relationship of mol% PC-36:4 (LL) and mol% TAG54:6 (LLL). (f) Relationship of mol% PC-34:1 (PO) and mol% TAG-52:3 (POL). (b,c) suggest association of seed oil content and fatty acid composition, while (d,e,f) support metabolic precursor-product relationships between PC species and TAG species.

The several genotypes analyzed here actually could be divided into “high linoleic (18:2)” (like E01-3) or “high oleic (18:1)” (like TX-2500) phenotypes (Figure 3), and these differences in overall fatty acid composition were reflected in the molecular species profiles of both PC and TAG (Figure 3). For example there was a strong relationship between PC 34:2 content and TAG 54:4 content; similarly, there was a strong relationship between PC34:1 and TAG 52:3—and these were associated with high linoleic and high oleic phenotypes respectively, even in the few selected species and genotypes examined here. These differences in lipid compositions suggest that there may be much wider variation than realized across diverse *Gossypium* genotypes in monounsaturated fatty acid content, and that *FAD2* gene expression/activity may represent a

good target for molecular marker-assisted breeding. Since high-oleic seed oils have meant price premiums or increased market share for other oilseed crops, like sunflower, safflower, soybean, and corn (Dyer *et al.*, 2008), this variation in *Gossypium* may represent a potentially valuable association for cotton breeders to examine more closely. Additional incentive may come from the positive relationship that was noted between oleic acid percentage and overall seed oil content. In other words, among these diverse genotypes with wide-ranging oil contents, there was a tendency for seeds that had the highest oil content to have the highest oleic acid content. By contrast, highest linoleic acid percentage was noted for those with lowest oil content, suggesting that those with lowest FAD2 activity in embryos tended to accumulate more seed oil. Certainly, this is not a simple relationship, and there are far too few accessions compared here to draw definitive conclusions, but it is an interesting relationship that may warrant further attention. It is worthwhile to note that the desaturation reaction by the FAD2 enzyme requires reducing equivalents and molecular oxygen, both of which if not required might prove for a more efficient overall accumulation of seed lipids.

IMAGING LIPID METABOLITES IN SITU IN COTTONSEEDS

Shotgun lipidomics is indeed a valuable tool for identifying and quantifying individual molecular species of lipid classes in cottonseeds; however, the location of these lipids within the embryos is lost during solvent-based extraction of seed tissues. Application of matrix-assisted laser desorption/ionization (MALDI)-MS imaging has recently been developed as a new technique to combine the high-resolution chemical information of mass spectrometry with the localization of these metabolites in tissue sections ((Horn *et al.*, 2012); see also Figure 4). Such studies have revealed surprising heterogeneity within cottonseed tissues (Horn *et al*., 2012; 2013)), and have suggested that this heterogeneity results from differences in the metabolic pathways that are involved in the assembly of PC and the production of TAGs at these different locations. Understanding the basis for this heterogeneity will provide important insights into the pathways and enzymes that are responsible for seed oil content and composition.

Based on the compositional diversity in seeds from these diverse genotypes, we suspected that there might be differences in the tissue localization of TAG and PC metabolites among some of these embryos. Indeed, variation in compartmentalization of these lipid metabolites was evident in the four genotypes examined (Figures 5, 6, 7, 8). These results suggest marked differences in the organization of seed lipid metabolism in these species.

The spatial distribution of gossypol and six different molecular species of PC are shown for embryos of four different *Gossypium* species in Figure 5. Images are reconstructed on a gray scale (black is highest concentration; maximum units are listed on the scale) on an absolute ion basis (for gossypol; Figure 5b) or on a mol fraction basis of the total lipid class (for PC species; Figures 5c-g). Two-dimensional images are constructed using software, Metabolite Imager, written for the conversion of MALDI-MS data to spatial images (Horn and Chapman, 2013). These chemical maps are oriented with bright-field images of tissue sections (Figure 5a), to provide the tissue-based context of metabolite distributions. For validation and by way of example of the

methodology, the spatial distribution of gossypol metabolites is associated with pigmented glands throughout the embryos of these *Gossypium* species, although *G.stocksii* with few glands had little gossypol (Figures 5a,b). Heterogeneity in phospholipid composition was obvious immediately. Some PC species were distributed throughout the embryo uniformly, whereas others varied on a mol% basis between cotyledonary and embryonic axis tissues. Further, these patterns were variable across genotypes. For example, PC-34:1 (palmitic and oleic acids at the sn-1 and -2 positions) was more prevalent in outer cotyledonary tissues of *G. hirsutum* accession SA-1254, similar in pattern (but at a higher amount) compared to *G. anomalum* accession B01-1. This was different from the *G. barbadense* cv Pima-S6, or the *G. stocksii*, which showed a more uniform distribution of this PC species throughout the cotyledons and embryonic axis. PC 34:2 was more prevalent in the embryonic axis tissues relative to cotyledons, but the Pima-S6 embryos showed the reverse distribution (Figure 5d). Further, PC 36:3 showed opposing distributions (cotyledons vs axis) in G. anomalum and G. stocksii embryos (Figure 5f). Together these differences in PC distribution suggest differences in pathway enzymes leading to the assembly of these phospholipids, and because of the precursor-product relationship between PC and TAG, these patterns are predicted to be reflected in tissue-based heterogeneity of seed oil compositions.

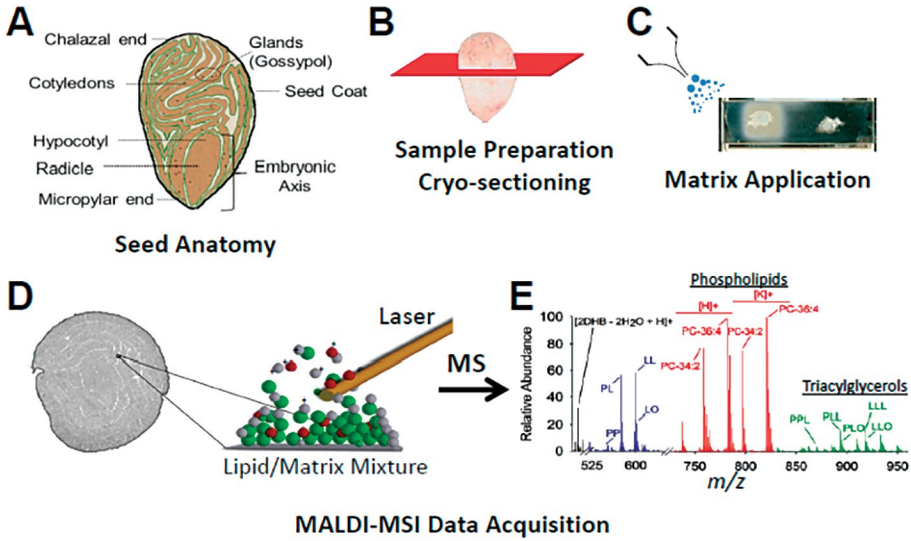


Figure 4. Schematic overview of MALDI-MS imaging process. (A) Longitudinal diagram of mature cotton embryo primarily composed of folded cotyledon tissues surrounding the embryonic axis (hypocotyl and radicle). (B) Embryos are sliced into thin cross- or longitudinal-sections with a cryostat. Sections of approximately 30 microns are coated with matrix (C) to promote the formation of ions. (D) A laser is rastered across the section at ~50 microns intervals. Lipid ions as [M + H]+, [M + Na]+, or [M + K]+ adducts are directed into a mass spectrometer (Thermo LTQ Orbitrap-XL). (E) Each detection cycle generates a raw data spectrum for lipid species at x, y position of the seed section. Images are reconstructed using Metabolite Imager (www.metaboliteimager.com). Legend and image modified from Horn et al. Plant Cell 2012 24: 622-636 with permission.

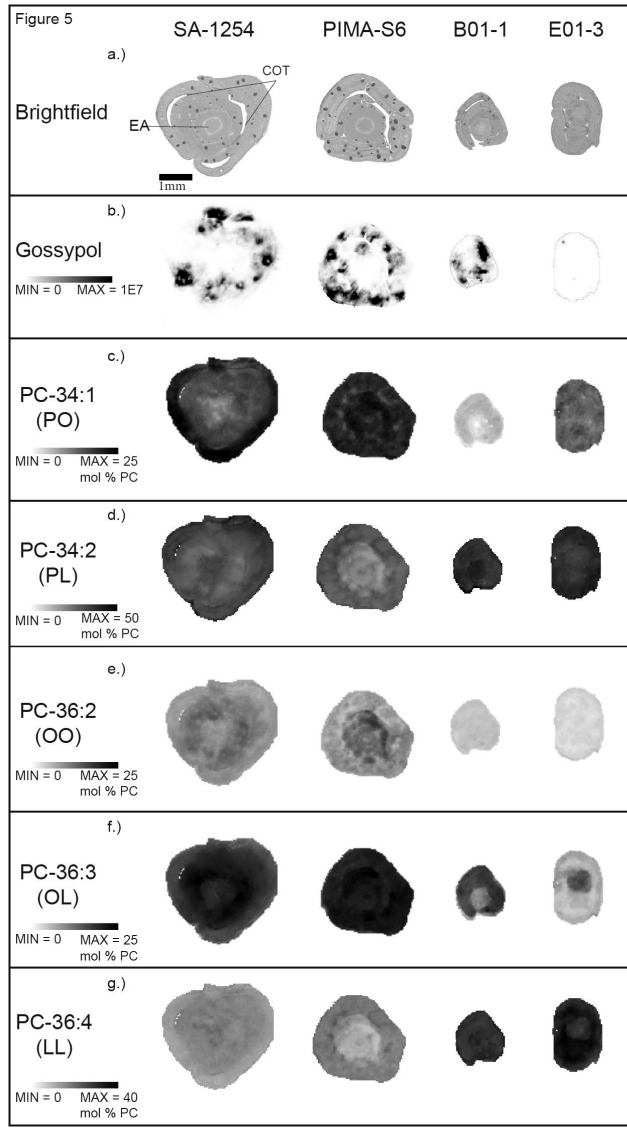


Figure 5. Images of lipid metabolites in cross-sections of embryos (middle) from *G. hirsutum* (SA1254), *G. barbadense* (Pima S6), *G. anomalum* (B01-1), *G. stocksii* (E01-1). Ion maps are generated from MALDI-MS for gossypol (b) and phosphatidylcholine (PC)molecular species (c-g), and compared to bright-field micrographs (a) for orientation. PC molecular species are denoted as total number of acyl carbons and number of total double bonds. (P is palmitic (16:0), O is oleic (18:1) and L is linoleic (18:2)). Gray scale images are converted from ion counts (b) or mol% of class (c-g) with black as highest.

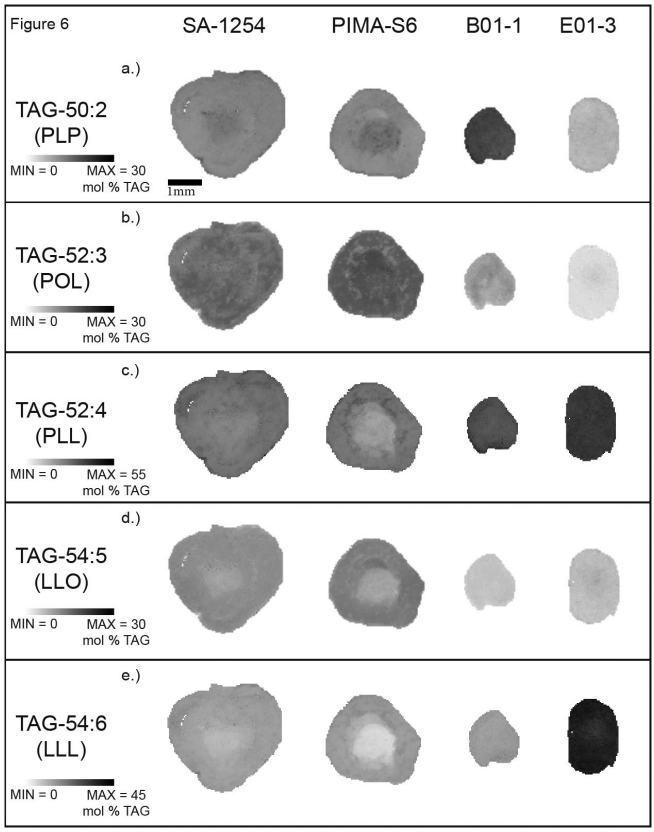


Figure 6. Images of lipid metabolites in cross-sections of embryos (middle) from *G. hirsutum* (SA-1254), *G. barbadense* (Pima-S6), *G. anomalum* (B01-1), *G. stocksii* (E01-1). Ion maps are generated from MALDI-MS for triacylglycerol (TAG) molecular species. Compare to bright-field micrographs (Figure 5a) for orientation. TAG molecular species are denoted as total number of acyl carbons and number of total double bonds. (P is palmitic (16:0), O is oleic (18:1) and L is linoleic (18:2)). Gray scale images are converted from mol% of class with black as highest relative amount.

The distribution of the major triacylglycerol species in embryos is shown in Figure 6 for four *Gossypium* species. Most obvious were the differences in the content of each of these TAGs in the different *Gossypium* backgrounds (e.g., the mol fraction of TAG-50:2, or PLP, was much greater relative to total TAG in B01-1embryos than in E01-3 embryos (Figure 6a). Although there were some heterogeneous distributions of TAGs evident within embryo tissues (like PLP, PLL, LLO in Pima S6; Figure 6), the large variation in amounts of each of the TAGs in the different *Gossypium* embryos masked somewhat the tissue-based heterogeneity within the species (Figure 6).

Differences in spatial distributions of TAG metabolites were considerable when comparing within a single *G. hirsutum* genotype (Horn *et al*., 2012; 2013), and this was also the case for these embryos when they are analyzed individually (Figure 7). Plots of TAG distributions in transects from left to right over embryo cross-sections showed that some TAGs were more concentrated in cotyledonary tissues than in the embryonic axis tissues (and some vice versa), and this was especially evident in *G. hirsutum* (SA-1254) and *G. barbadense* (Pima-S6). For example, TAG-52:4 (PLL) and TAG54:6 (LLL), were present at higher mol fractions of the total TAGs in cotyledons compared to the axis (Figure 7). These differences in distribution were not obvious for the smaller seed species, *G. anomalum* (B01-1) or *G. stocksii* (E01-3), and may reflect differences in TAG metabolic pathways among these diverse species.

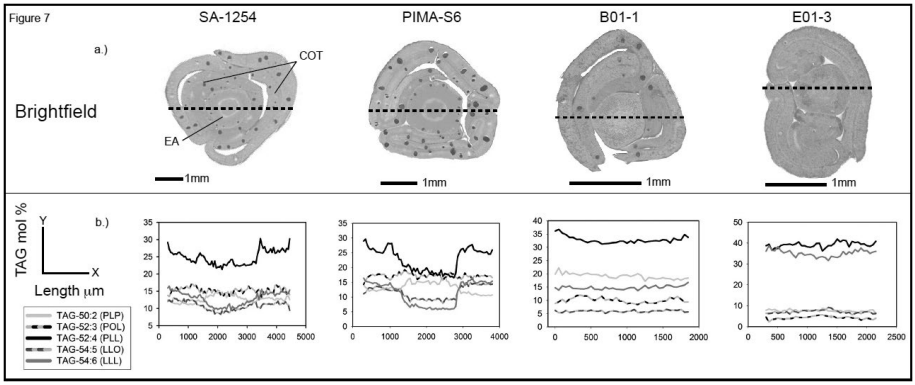
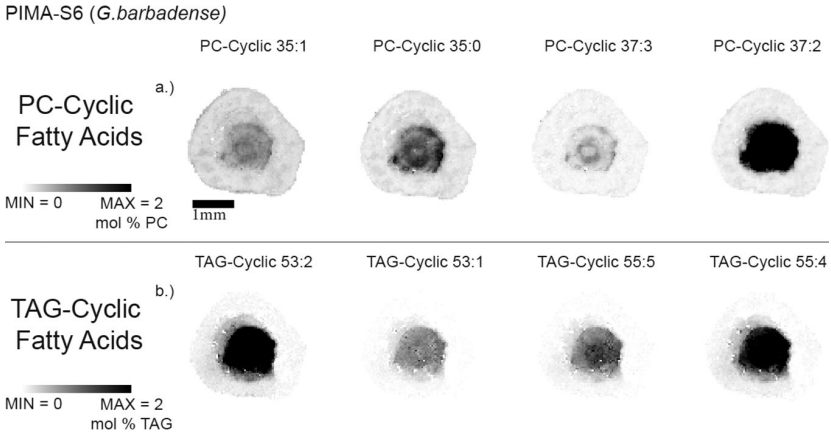


Figure 7. Triacylglycerol (TAG) distributions in transects across embryos of four *Gossypium* species. (a) Bright-field images of cross sections through embryos with the embryonic axis (EA) and cotyledon (COT) tissues labeled. (b) Plots of mol% values for five major TAGs in tissue sections crossing from left to right through cotyledon, axis and cotyledon tissues. Substantial spatial differences in distributions of TAGs were evident in *G.hirsutum* (SA-1254) and *G. barbadense* (Pima-S6), but were not so apparent in *G. anomalum* (B01-1) or *G. stocksii* (E01-3).

To illustrate the marked heterogeneity and metabolic relationship of PC and TAG metabolites within a species, the distribution of several PC and TAG species with a cyclic fatty acid are shown for embryos of *G. barbadense* cv Pima-S6 (Figure 8). Consistent with previous results for *G. hirsutum* embryos (Horn *et al*., 2012; 2013), the PC and TAG molecular species with a cyclopropane/ene fatty acid are restricted to the embryonic axis regions of the *G. barbadense* embryo. The metabolic relationships are consistent with this distribution since the cyclic group is introduced into the acyl chain when it is esterified to PC and these acyl groups on PC are utilized for the synthesis of TAGs. PC-cyclic 35:1 and PC-cyclic 35:0 are precursors for TAG-cyclic 53:2 and TAG-cyclic 53:1, respectively (in each case adding an 18:1 acyl group); the PC-cyclic 37:3 and PC-cyclic 37:2 are precursors for TAG-cyclic 55:5 and TAG-cyclic 55: 4 (in each case adding an 18:2 acyl group). So the heterogeneous distribution of these metabo-

lites suggests that the cyclopropane fatty acid synthase is mostly active in the embryonic axis and that the production of TAGs in these tissues may vary from that in cotyledonary tissues, at least in this respect. Since this pattern of cyclic fatty acid distribution holds across two diverse *Gossypium* tetraploid species, it suggests some fundamental importance in the *Gossypium* evolutionary history, perhaps as defense compounds during radicle emergence and early seedling growth (Liu *et al*., 2009; 2012).



SUMMARY

The domestication and breeding of cotton for elite, high-fiber cultivars has led to reduced genetic variation of seed constituents within currently cultivated accessions. A screen of the genetically diverse U.S. National Cotton Germplasm Collection identified accessions/species with dramatic differences in seed oil and protein content. Several genotypes were analyzed for quantitative and spatial differences in seed lipid compositions by mass spectrometric approaches. Results indicated marked variation in pathway metabolites for triacylglycerol biosynthesis in embryos across *Gossypium* species, and suggest that this variation might be exploited by breeders for seed composition traits. Given the large amounts of cottonseed produced in the U.S. that is currently not processed into higher value products, these efforts might be one avenue to raise the overall value of the cotton crop for producers. More comprehensive profiling of seed lipid metabolites across the *Gossypium* genus would appear to be warranted.

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