Title: Three-Dimensional Visualization of Membrane Phospholipid Distributions in *Arabidopsis thaliana* seeds: A spatial perspective of molecular heterogeneity

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Abstract:

Arabidopsis thaliana has been widely used as a model plant to study acyl lipid metabolism. Seeds of *A. thaliana* are quite small (approximately 500 x 300 µm and weigh ~20 µg), making lipid compositional analyses of single seeds difficult to achieve. Here we have used matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) to map and visualize the three-dimensional spatial distributions of two common membrane phospholipid classes, phosphatidylcholine (PC) and phosphatidylinositol (PI), in single *A. thaliana* seeds. The 3D images revealed distinct differences in distribution of several molecular species of both phospholipids among different seed tissues. Using data from these 3D reconstructions, the PC and PI mol% lipid profiles were calculated for the embryonic axis, cotyledons, and peripheral endosperm, and these data agreed well with overall quantification of these lipids in bulk seed extracts analyzed by conventional electrospray ionization-mass spectrometry (ESI-MS). In addition, MALDI-MSI was used to profile PC and PI molecular species in seeds of wild type, *fad2-1*, *fad3-2*, *fad6-1*, and *fae1-1* acyl lipid mutants. The resulting distributions revealed previously unobserved changes in spatial distribution of several lipid molecular species, and were used to suggest new insights into biochemical heterogeneity of seed lipid metabolism. These studies highlight the value of mass spectrometry imaging to provide unprecedented spatial and chemical resolution of metabolites directly in samples even as small as a single *A. thaliana* seeds, and allow for expanded imaging of plant metabolites to improve our understanding of plant lipid metabolism from a spatial perspective.

Keywords:

3D, MALDI-MSI, Lipids, *Arabidopsis*, Phosphatidylcholine, Phosphatidylinositol

Abbreviations:

Arabidopsis thaliana (*A. thaliana*); *Camelina sativa* (*C. sativa*); *Gossypium hirsutum* (*G. hirsutum*); *Brassica napus* (*B. napus*); Triacylglycerol(s) (TAG(s)); Phosphatidylcholine (PC); Phosphatidylinositol (PI); Matrix assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI); Electrospray ionization-mass spectrometry (ESI-MS). Numerical designations for molecular species is total number of carbons in acyl chains: total number of double bonds.

1.Introduction:

In oilseeds, the metabolic pathways leading to the synthesis and accumulation of storage triacylglycerols (TAGs) are surprisingly complex, involving both acyl CoA-dependent and acyl CoA-independent acyltransferases [1]. However, both of these routes utilize the common membrane phospholipid, phosphatidylcholine (PC), indirectly or directly, as precursor molecules for TAG assembly. Acyl groups esterified to PC are the substrates for the ER bound desaturases [2], meaning all polyunsaturated fatty acids in oilseeds are synthesized via a PC intermediate. The phosphocholine head group of PC may be removed to generate a so-called, PC-derived diacylglycerol as a substrate for TAG synthesis; or, alternatively, the acyl groups on PC can be transferred directly into a TAG via the phospholipid:diacylglycerol acyltransferase (PDAT) enzyme [3]. Therefore, in addition to the well-known role of PC molecular species as structural components of membrane bilayers, these phospholipids are key metabolic precursors for the synthesis of oils in seeds, and the detailed compositional analyses of PC molecular species in seeds provide important insights into the regulation of seed lipid metabolism. In addition, the capacity for PC and TAG synthesis appears to be important for aspects of proper embryo development, since loss of function in genes that are involved in synthesis of PC precursors (*SDC1*, *CEK4*, *PECT*), assembly of PC (*AAPT1/AAPT2*), or assembly of TAG (*PDAT1/DGAT1*) results in embryo lethality [4-8], emphasizing the importance of PC and TAG metabolites to reproductive development and early stages of seed formation.

Arabidopsis thaliana, a close relative of the oilseed crops, *Brassica napus* and *Camelina sativa*, is a widely used model plant to probe the biochemistry of lipid metabolism and to test the capacity for its manipulation in seeds. *A. thaliana* was used in the 1990s to identify and characterize many of the genes involved in glycerolipid synthesis and acyl-chain desaturation and elongation [9-12]. The many resources available for *A. thaliana* and its prolific and rapid reproduction in a relatively small space have made the use of *A. thaliana* commonplace for genetic and biochemical studies of metabolism. While the small, albeit large numbers of, seeds are advantageous for genetic studies, this has placed limits on the spatial considerations of biochemistry within *A. thaliana* individual seeds and tissues (e.g. endosperm, embryonic axis, radicle or cotyledons). Instead, bulk extractions and analysis of numerous seeds have been used most often to draw conclusions about seed lipid metabolism, and this tissue extraction disrupts any original information about the specific location of metabolites. Recent information about localized heterogeneity of lipid metabolites in other plant systems [13, 14], and the spatial-developmental differences in transcriptome profiles within *A. thaliana* seeds [15], have

motivated our efforts here to visualize the spatial distribution of membrane lipids in individual *A. thaliana* seeds.

Advances in techniques to visualize the spatial distribution of metabolites in cells and tissues have the potential to markedly change our understanding of plant lipid metabolism. Magnetic resonance imaging (MRI), where the signals from nuclear spins of atoms in target classes of molecules are detected and spatially reconstructed into three-dimensional images[16], have been used to visualize the distribution of lipids in seed tissues, including the oilseeds, *C. sativa*, *B. napus* and *G. hirsutum* (upland cotton) [17-19]. However, while imaging by NMR provides a non-destructive, detailed three-dimensional view of metabolites *in situ*, this MRI of plant tissues remains somewhat constricted by the nature and size of the sample, and it cannot yet distinguish between different lipid classes or different molecular species of lipid metabolites *in situ*. One popular, alternative technique for revealing detailed metabolite composition information *in situ* is matrix assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI). MALDI-MSI applications in plant tissues allow for the direct visualization of metabolites *in situ* with high chemical and spatial resolution [14]. Recently, the cellular/subcellular investigation of *Zea mays* leaves in transverse cross sections demonstrated the differential localization of flavonoids in the single cell layer of upper and lower epidermis [20]. Additionally, they distinguished differences in heterogeneity of phosphatidylglycerol (PG) molecular species between the bundle sheath and mesophyll cells. Elsewhere, more than fifty membrane and storage lipid molecular species were visualized *insitu* in seeds of *C. sativa* and *G. hirsutum* and were shown to be localized in a heterogeneous manner [17, 18, 21]. The heterogeneous localization of plant lipid metabolites suggest that important unappreciated differences in the spatial organization of plant metabolism remain to be discovered and that future attempts to reconcile metabolite distributions with transcriptional and posttranscriptional regulatory mechanisms are warranted.

Two important advances in plant metabolite imaging are presented here that will likely facilitate the future integration of metabolite location information with larger scale "omics" data. First, we present a means to map in three dimensions, the detailed phospholipid species distribution for two important phospholipid classes, phosphatidylcholine (PC) and phosphatidylinositol (PI), in seeds of *A. thaliana* at approximately 15x15x20 micron spatial resolution. Second, we demonstrate that genetic mutants in lipid metabolism show unexpected changes and differences in the distribution of membrane lipids in seeds, and are unlikely to be entirely accounted for by transcriptional mechanisms. Our results collectively suggest that

additional, spatial perspectives in metabolic regulation should be considered to unravel the overall complexity of metabolism in plant systems at the organismal level.

2. Methods:

2.1 Chemicals

Optima grade isopropyl alcohol (IPA), chloroform (CHCl_3), methanol (MeOH), and butylated hydroxytoluene (BHT), were purchased from Sigma-Aldrich (St. Louis, MO, USA) in Optima grade. Water was 18 M Ω and generated in house. Chemicals 1,5-diaminonaphthalene (DAN, 97%), 2,5-dihydroxybenzoic acid (DHB, 98%) and gelatin from porcine skin (300 bloom) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Plant Materials

Seeds for MALDI-MS imaging and lipid extractions were harvested from plants of *Arabidopsis thaliana* (Columbia-0) wild type, *fad2-1* (CS8041; At3g12120), *fad3-2* (CS8034; At2g29980), and *fae1-1* (CS6245; At4g34520) grown in chambers maintained at 21°C with a 16:8 light:dark cycle and 50 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}$ light intensity. Seed stocks were originally acquired from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Seeds of the *fad6-1* (CS207; At4g30950) mutant were graciously supplied by Dr. John Browse at Washington State University and were embedded for sectioning upon receipt.

2.3 Cryo-sectioning for MALDI-MS Imaging

Desiccated seeds were embedded in a 10% (w/v) gelatin solution that had been prepared and allowed to equilibrate in a 40°C water bath with gentle shaking for a minimum of two hours. Seeds, embedded in gelatin, were transferred to a -20°C freezer for 5 min to solidify, frozen at -80°C for 1-2 hours, then equilibrated for a minimum of 12 hours at -20°C before sectioning. Embedded seeds were sectioned using a cryo-microtome (Leica CM1850, Leica Microsystems; Buffalo Grove, IL, USA) set to a temperature of -17°C and cut to a section thickness of 20 μm . Sections were collected on charged glass slides (Fisher Scientific; Waltham, MA, USA) by thaw mounting, then placed on a chilled (-80°C) aluminum block and lyophilized under moderate vacuum (~250 mtorr) for 1-1.5 hours. The lyophilizer was then slowly equilibrated to room temperature and atmospheric pressure (760 Torr), and sections were stored in a desiccator until matrix application for MALDI-MS imaging. All sections were imaged within 36 hours of

sectioning. Identification of appropriately-oriented and serially-sectioned *A. thaliana* seeds made possible the reconstruction of three-dimensional seed images following MALDI-MSI.

2.4 MALDI Mass Spectrometry Imaging Analysis

Both matrices used here, 2,5-dihydroxybenzoic acid (DHB) and 1,5-diaminonaphthalene (DAN) were applied to serial sections by sublimation using a procedure adapted from Hankin et al.[22]. MS imaging data were collected using a MALDI-linear ion trap (LIT)-Orbitrap mass spectrometer (MALDI-LTQ-Orbitrap Discovery, Thermo Scientific; San Jose, CA, USA). The instrument was modified to use an external 355nm Nd:YAG laser (UVFQ, Elforlight Ltd.; Daventry, UK) and a custom optical set-up. Laser optics are similar to previously described [20] but a commercial 10X beam expander (Thorlabs, Newton, NJ, USA) was used which reduced the laser spot size to 13-15 μm with 20 laser shots. TunePlus and Xcalibur software (Thermo Scientific) were used to define image and other data acquisition parameters. The laser energy used was 83-85% (0.4-0.7 $\mu\text{J}/\text{pulse}$) at a 60 Hz repetition rate. MS imaging data were acquired using 15 μm raster step size and 20 laser shots per step. Mass spectra were acquired in positive and negative mode using the Orbitrap mass analyzer with a resolution of 30,000 and m/z scan range of 500-1200.

2.5 Processing Data for MALDI-MSI

Raw mass spectral files acquired from the Orbitrap analyzer were used to generate images using ImageQuest software (Thermo Scientific; San Jose, CA, USA). Each MALDI mass scan represents an absolute position (e.g., with x, y coordinates) analyzed within the seed section. Individual scans, at a particular x, y positions, were processed to search for membrane phospholipid molecular species with a theoretical m/z with width of ± 0.003 . This tolerance was selected based on the high resolving power of the Orbitrap analyzer and refined through rounds of m/z analysis of peaks identified. Processed MALDI-MS images were normalized to the total ion count for each pixel. Lipid mol% profiles in MALDI-MS images were calculated using MetaboliteImager [23], and were relative to the total lipid class in the selected tissue area in 2D x-y scans or in 3D reconstructions (multiple, stacked 2D scans). Molecular ions were identified by accurate mass, and were confirmed by MS/MS analysis on sections and in extracts. For relative quantification of PC and PI in two dimensions, values were averaged from three different sections (three different seeds) selected from approximately the same location and orientation in the embryo. For the 3D models, relative mol% was calculated for each serial section of a single seed before assembly into a 3D image.

2.6 Development of 3D models

Three-dimensional MALDI-MSI reconstructions were accomplished by collecting MALDI-MSI data sets for consecutive, serial sections of a sample, and reconstructing 3D images of each analyte from the multiple, stacked MALDI-MS images. Image processing software packages including ImageQuest, and ImageJ were used to generate 3D images. 2D profile images of each specific m/z were produced using ImageQuest in '.TIFF' format, and stacked together using TrakEM2 module [24-26] of ImageJ (version 1.50e). Each 2D image was placed on top of the previous image, and adjusted for x, y position and rotation to properly orient the section relative to the previous sections. To ensure proper orientation and alignment, the 2D images were positioned using a half-transparent overlap with the previous image. Once the 2D MALDI MS images were stacked, aligned and transformed, ImageJ was used to display a 3D model and create 3D MALDI-MS image videos.

2.7 Lipid Extraction

Total lipids were extracted from 5.0 mg of *A. thaliana* seeds of all genotypes as described by Chapman and Moore [27] with a few modifications. Seeds were ground in 1.0 mL of hot (70°C) IPA with 0.01% butylated hydroxytoluene (BHT) (w/v) using glass beads in a bead beater (Biospec Mini-Bead-Beater-16, Bartlesville, OK, USA). An additional 1.0 mL of hot IPA was added to these extracts and incubated in a 70°C water bath for 30 mins to inactivate endogenous phospholipases. After incubation, CHCl_3 and water were added to a ratio of 2:1:0.45 (v/v/v) IPA: CHCl_3 : H_2O and 20 μg of PC (di-14:0) and 3.0 μg PI (di-16:0) internal standards were added to each sample. Samples were extracted overnight at 4°C, and then were partitioned into the organic phase by adding 1.0 mL of CHCl_3 and 2.0 mL of 1.0 M KCl. Total lipid extracts (TLE) in the organic layer were purified with three washes 1.0 M KCl (2.0 mL each time). Purified total lipid extracts in chloroform were dried under a stream of nitrogen gas and re-suspended in 1.0 mL of 2:1 (v/v) MeOH: CHCl_3 .

2.8 Direct Infusion ESI-MS Lipid Profiling

TLEs were analyzed by direct-infusion ESI-MS on a triple quadrupole mass spectrometer (API 3000; Applied Biosystems, Foster City, CA, USA) according to [28]. Samples for ESI-MS analyses were prepared by evaporating the solvent from 100 μL TLE to dryness and resuspending in 1.0 mL of MeOH: CHCl_3 :300 mM ammonium acetate (665:300:35, v/v/v). MS data acquisition conditions were as follows: a direct infusion rate of 20 $\mu\text{L}/\text{min}$, capillary voltage of +5.5 kV, source temperature 100°C, and the nebulizing and curtain gases were set to 12 and 8 (arbitrary units), respectively [28]. PC molecules were detected as protonated, $[\text{M}+\text{H}]^+$, using

the precursor scan mode set to detect all the precursor ions of a fragment m/z 184 (PC head group), with entrance and exit potentials set to 14V and collision energy of 40V. PI molecules were detected as ammoniated, $[M+NH_4]^+$, using the neutral loss scan mode set to detect all the ions with the neutral mass loss of 277 Da (PI head group + NH_3), with entrance and exit potentials set to 14V, and collision energy 25V.

Subsequent product scans in negative ionization mode were used to confirm acyl chain composition (but not stereospecific position on the glycerol backbone) for each molecular species of PC and PI (Table 1). In negative ion mode, PC molecules were detected as acetate adducts, $[M+OAc]^-$, whereas PI molecular ions were detected as deprotonated, molecules $[M-H]^-$ [29, 30]. Collision energies between 25-50V were used to fragment parent ions of each molecular species of PC and PI into their respective daughter ions, from which acyl chain fragment ions could be identified. ESI-MS data collected were exported as text files using Analyst software (Applied Biosystems) and analyzed using LipidomeDB [31]. Mol% values of each species within a given class were quantified after calibration with the internal standards in ESI-MS, and these profiles were compared with the relative mol% values summed over sections imaged *in situ*.

3. Results:

3.1. Lipid Profiles in Three Dimensions in *A. thaliana* Seed Tissues

The overall workflow for specimen preparation and MALDI-MS imaging is presented in Fig. 1. Wild-type *A. thaliana* (ecotype Columbia-0) seeds were sectioned in longitudinal or transverse cross-sectional orientations (Fig. 2A and 2B, respectively). Mature seed tissues and cell types were identifiable in bright-field microscopy, including those of the thin peripheral endosperm, two cotyledons and embryonic axis (hypocotyl and radicle tissues). Serial sections of 20 microns in thickness were collected through the entire embryo and seed coat, from one side to the other side (Fig. 2C) or from top to bottom (Fig. 2D), resulting in 14 longitudinal sections side-to-side or 25 transverse, cross-sections from chalazal to micropylar ends of the seeds. Positive mode MALDI-MSI data sets were acquired for longitudinal sections with DHB as a matrix, and negative mode data sets were acquired for transverse sections with DAN as a matrix.

MALDI-MS images were generated for PC molecular species in all serial sections of positive mode data sets. Here, the spatial distributions of three PC molecular species are presented that showed particularly heterogeneous localizations in the different seed tissues (Fig. 3). The MS images for each serial section were stacked as described in methods to yield a three-dimensional view of each PC molecular species in the seed (Figs. 3D, 3E, and 3F). False-colored images for each molecular species were adjusted to the same maxima for each species where red was most abundant and green was least abundant. Based on ion intensity normalized to total ion count (TIC), PC 34:2 at m/z 758.571 for $[M+H]^+$ was enriched in the embryonic axis tissues in all serial sections through the embryo (Fig. 3A and 3D) whereas PC 34:3 at m/z 756.554 for $[M+H]^+$ was enriched in the peripheral endosperm layer (Fig. 3B and 3E). By contrast, PC 38:3 at m/z 812.617 for $[M+H]^+$ (Fig. 3C and 3F) was enriched in all serial sections in the tissues of the cotyledons, opposite of the embryonic axis and of the shorter chain PC 34:2 species. It should be emphasized that these distributions were similar in each serial section, and so provide confidence in the conclusions of their heterogeneous distributions, and replicate sections from different seeds revealed consistent distributions. Video animations of the 3D distributions for these PC molecular species allow for rotation and visualization of the seed and seed tissues (see supplemental videos 1-3). Because PC is the major intermediate in the metabolism and shuttling of acyl groups into triacylglycerols (TAGs)[1], the striking differences in localization of these PC molecular species within the seed tissues may reflect the metabolic demands for TAG synthesis in different tissues. Or, there may be specific structural

requirements/functions for certain PC species in these different tissues. In any case these differences in the distributions of PC molecular species raise intriguing questions about the extent of heterogeneity of lipid metabolism pathways in seeds.

In addition to PC, we questioned whether other membrane phospholipids, especially those not involved in TAG biosynthesis, were distributed in a heterogeneous manner in *A. thaliana* seeds. Here, we examine the phospholipid class PI which preferentially ionizes in negative ionization mode. Consequently, we analyzed seed tissue sections by MALDI-MS in negative mode with DAN as a matrix. MALDI-MS images were produced for PI molecular species in all serial sections, and the spatial distributions of two species differentially localized in seed tissues are presented in Figure 4 as examples. The MS images for each serial section were stacked as described in the methods to yield a three-dimensional view of each PI molecular species looking from the chalazal end of the seed through to the micropylar end (right panels). False-colored images were adjusted to the same maxima throughout the sections for each species where red was most abundant and blue was least abundant. Based on ion intensity normalized to TIC, PI 34:2 at m/z 833.518 for $[M-H]^-$ was enriched in the embryonic axis tissues in all serial sections through the embryo (Fig. 4A and 4C). In sharp contrast, PI 36:3 at m/z 859.533 for $[M-H]^-$ (Fig. 4B and 4D) was enriched in the tissues of the cotyledons, opposite to the embryonic axis and of the shorter chain PI 34:2 species. As with PC species, these heterogeneous distributions were similar in each serial section, and so provide confidence in the conclusions of their spatial differences. Video animations of the 3D distributions for these PI molecular species allow for rotation and visualization of the seed and seed tissues (see supplemental videos 4, 5). Ion intensities in the videos are displayed on a blue to red scale, where the red represents the maximum intensity. Because acyl groups in PI are not generally considered to be intermediates for the synthesis of TAGs, the heterogeneous distribution of these two PI molecular species are likely attributed to some functional significance other than storage lipid metabolism, possibly signaling or membrane stability.

These 3D reconstructions of lipid molecular species provide unprecedented spatial and chemical resolution of the lipidome in single *A. thaliana* seeds. In addition to mapping the spatial localization of metabolites, here we also used 3D MALDI-MSI data to develop a semi-quantitative estimate of the relative mol% of each species in a whole single seed. Analyses by MALDI-MS are typically considered semi-quantitative because multiple factors can interfere with accurate quantification such as in-source fragmentation, multiple adduct formation, and differences in ionization efficiencies across different classes of molecules. On the other hand, in

our experience there has been good experimental agreement between relative quantification of molecular species within the same lipid class (*i.e.*, mol% within each lipid class) by MALDI-MSI and extract analysis by conventional shotgun lipidomics [13, 17, 18]. However, our previous comparisons may have had some limitations since they were calculated from the 2-dimensional distribution of the molecular species in a single cross-section which, because of the noted heterogeneity, may not be representative of its distribution within the whole seed. Here we estimated the relative quantification in mol% of molecular species for PC and PI by assembling data from serial scans, and compared these values that were visualized in a single seed with percentages that were quantified from whole seed extracts. In other words, we used the MALDI-MS data to reconstruct an estimate of the 3-dimensional distribution of the selected species in the embryo, and used the extract data to help validate the accuracy of the overall relative quantification.

We constructed 3D representation of mol % of the whole seed in two different ways as illustrated in Figure 5A. In the first approach, the mol% of the lipid molecular species were calculated for each section, multiplied by the number of voxels in that section, and then summed together across all the tissue sections before normalized by the total number of voxels for the whole seed. In the second set of calculations, the raw ion intensities for each lipid molecular species were summed together across the tissue sections, then the relative mol% was calculated by normalizing to the total ion signals from the same lipid class. The results were nearly identical between the two methods for the major molecular species of both PC (Fig. 5D) and PI (not shown). Furthermore, these results were in excellent agreement with the direct infusion ESI-MS analysis of total lipid extracts (Fig. 5E and 5F). Minor differences may result from comparing the mol% distribution of a single seed (MALDI-MSI) with those from extracts (ESI-MS) of ~250 seeds. Nonetheless, agreement of relative quantification values between 3D MALDI-MSI and total lipid extracts suggests that the distributions visualized *in situ* accurately reflect endogenous lipid profiles. This indicates that the unadjusted ion intensity alone can be used in a semi-quantitative manner to calculate the relative-mol% of these lipid molecular species within a given class-especially if the experimental conditions were consistent across the sections. Most importantly, the 3D MALDI-MSI data sets have the advantage over total extract analysis in that the summed mol% values can allow for the visual evaluation of distributions for individual lipid metabolites in different seed parts (embryonic axis, cotyledons, and peripheral endosperm) simply by considering how many voxels are occupied by each tissue type (Fig. 5E and 5F). Essentially, this corresponds to a virtual dissection experiment where the lipid

composition of each seed part can be localized and visualized without time consuming actual dissections.

3.2. Changes in Molecular Heterogeneity in Acyl Lipid Mutants

In an effort to better define the molecular mechanisms involved in establishing heterogeneity in phospholipid distribution in *A. thaliana* seeds, a number of mutants in acyl lipid desaturation (*fad2-1*, *fad3-2*, *fad6-1*) and elongation (*fae1-1*) were evaluated by 2D MALDI-MS imaging (Figs. 6-8). The distributions of the principal molecular species of PC (Figs. 6 and 7) and PI (Fig. 8) were compared between seeds of wild-type (Col-0) and these mutants, all in the Col-0 genetic background. For MALDI-MSI analyses (Figs. 6-8), the ion intensities are shown using a blue to red scale, with red representing the highest intensity. Intensities were adjusted for the 34C and 38C species of PC to reveal heterogeneity of lower abundant species, and these scales are provided directly on the appropriate Figs. Distributions of all lipid molecular species of PC and PI in all acyl-lipid mutants were confirmed in three or more biological (seed) replicates. Relative quantification of lipid mol% from MALDI-MS images and conventional quantification (ESI-MS) of lipid species against internal standards in total lipid extracts are provided for comparison for each lipid class.

For 34C and 38C PC species (Fig. 6) and the 36C PC species (Fig. 7), the most striking differences from wild type seeds were noted for the *fad2-1* (Figs. 6B and 7B). Most of these changes of *fad2-1* were attributable to a change in fatty acid composition, a reduction in 18:2 and 18:3 fatty acids and an increase in 18:1 fatty acids, generating new predominant 18:1 containing molecular species (e.g. PC 34:1, PC 38:2, and PC 36:2). MS/MS data presented in Table 1 also confirmed 18:1 fatty acid was a major component of PC 34:1, PC 38:2, and PC 36:2 in *fad2-1*. In *fad2-1*, these new predominant PC molecular species exhibited varying degrees of heterogeneity; PC 38:2 was localized preferentially in cotyledon tissues, whereas PC 34:1 and PC 36:2 were distributed relatively homogeneously throughout the cotyledons and embryonic axis tissues. Here, a loss-of FAD2 function was clearly responsible for the increase in 18:1 containing species (quantified in Fig. 6F-G and Fig. 7F-G) as expected, but other mechanisms were apparently responsible for the differential localization of these “newly dominant” species. It is worth pointing out here, that the loss of *fad2-1* did not eliminate the localization of PC 34:3 from peripheral endosperm tissue in the *fad2-1* mutant, suggesting that perhaps other desaturases were responsible for the formation of polyunsaturated fatty acids in these cell types. Similarly, a small but significant amount of PC 34:2, PC 36:4 and PC 36:3 species were formed in the *fad2-1* seeds, but this was mostly restricted to the peripheral

endosperm and tissues in the radicle tip, suggesting that there may be an alternative route for the synthesis of 18:2 and 18:3 in these tissues. An alternative desaturase may be FAD6, in at least in the case of PC 36:3, since the *fad6-1* mutant lost this localization of PC 36:3 in the radicle tip. However, FAD6 does not seem to account for the PC 34:2 and PC 36:4 distributions, because the axis/radicle localization of these molecular species were not different from wild type in *fad6-1* mutants (compare Fig. 6D with 6A, 6B and Fig. 7D with 7A, 7B).

There were fewer differences between *fad3-2* and wildtype seeds in terms of PC 34C and 38C molecular species (Fig. 6C). However, levels of PC 34:3 were reduced in the cotyledons and embryonic axis, but remained present in the peripheral endosperm. This was similar to the peripheral endosperm localization of polyunsaturated PC molecular species in *fad2-1*, suggesting a similar pathway of desaturation, possibly FAD 7/8 [32]. Additionally, in the *fad3-2* mutant PC 36:5 was almost completely absent with only trace amounts in the peripheral endosperm as might be expected due to a reduction in 18:3 fatty acid. Furthermore, there was also a more uniform distribution of PC 36:3 throughout embryo tissues in the *fad3-2* mutant compared with wild-type (Fig. 7C vs 7A).

For *fae1-1* loss-of-function mutants, the changes in PC molecular species were primarily observed by the lack of the 38C species (Fig. 6E), in agreement with the fact that 20:1 acyl group, the elongated fatty acid product of FAE1, is needed for 38C species (Table 1). Few other differences in PC localization in *fae1-1* mutants were noted, suggesting this protein most likely influences cotyledon enrichment of 20:1-containing PC species through its expression in those tissues. For all wild-type and mutant seeds analyzed, the mol% lipid profiles of PC molecular species were consistent between MALDI-MS and ESI-MS of extracts (Fig. 6F-G and Fig. 7F-G). Identities of all PC molecular species in all genotypes were confirmed by MS/MS in extracts of seeds (Table 1).

Evaluation of the profiles of the principal PI molecular species in the acyl lipid mutants versus wild-type seeds (Fig. 8) was undertaken to ask how heterogeneity might be influenced in a membrane lipid that was not involved in storage lipid metabolism (like PC). Sections were obtained in the cross-sectional orientation and were visualized by MALDI-MS in negative ion mode with DAN as the matrix. PI 34:2 was the major species identified in wild-type and was distributed preferentially in embryonic axis tissues (top) relative to cotyledonary tissues (bottom) (Fig. 8A). This heterogeneous distribution was similar to that for PC 34:2, indicating that mechanisms for restricting the distribution of PC species may also influence the diacylglycerol portion available for the synthesis of other membrane lipids like PI. Mutations in FAD2

generated new predominant 18:1 containing species in PI (Fig. 8B), resulting in a more abundant PI 34:1, that had a uniform distribution between cotyledons and axis, and PI 36:2 that was more preferentially distributed in cotyledons relative to the axis. It is also of interest to note that PI 34:2 was still detected in peripheral endosperm and axis tissues in *fad2-1* seeds, again suggesting that alternative means of desaturation of 18:1 to 18:2 exist in these tissue types. These altered molecular species compositions and the spatial localizations were consistent with the likely available diacylglycerol moieties from PC for PI synthesis (compare Figs. 6B, 7B with Fig. 8B), despite the block in acyl lipid desaturation in PC (lack of FAD2 activity) [10]. The distribution and predominance of PI 34:2 did not change in other acyl lipid mutants compared to wild type seeds. In *fad3-2*, PI 34:3 was dramatically reduced with the exception of minor amounts in the peripheral endosperm, similar to PC 34:3 and PC 36:5 (Figs. 6C, 7C, and 8C). The seeds of mutants for *fad6-1* and *fae1-1* were not different from wild type in their PI species compositions/ localizations (Fig. 8D-E), reflecting the relative lack of these gene products in the contributions to the major PI species in *A. thaliana* seeds. The relative mol% lipid distributions for PI species were calculated from MALDI-MS images of the acyl-lipid mutants, and compared to wild-type (Fig. 8F). Overall the PI mol% lipid profiles obtained from MALDI-MS were in good agreement with the quantification of these same species by ESI-MS analysis (Fig. 8G). Furthermore, the acyl-chain compositions of each PI molecular species determined from MS/MS analysis of the lipid extracts and supported in identification of the PI molecular species (Table 1).

4. Discussion:

4.1 Three- Dimensional Visualization of the Lipidome in *A. thaliana* Seeds

In this study, the three-dimensional spatial complexity and heterogeneous distribution of a number of membrane phospholipids in individual *A. thaliana* seeds has been fully resolved for the first time using MALDI-MS imaging. At near cellular resolution, we mapped major and minor molecular species of PC and PI localized in the embryonic axis, peripheral endosperm and cotyledonary tissues in three dimensions, revealing remarkable differences in phospholipid distributions among these different seed parts. Here we have focused on two membrane lipid classes—one, PC, that is both a structural lipid and a critical metabolite in TAG metabolism in these seed tissues, and another, PI, that functions outside of TAG metabolism as a structural membrane lipid and a precursor for polyphosphoinositide signaling lipids. This work will facilitate future applications of MALDI-MS imaging for other phospholipid classes, such as TAGs, PG, phosphatidylserine (PS), phosphatidylethanolamine (PE), mono- and di-galactosyl diacylglycerols (MGDG and DGDG), sulfoquinovosyl diacylglycerol (SQDG), and others. Most of these lipid classes have been imaged in seeds and/or other plant tissues [13, 14, 20] illustrating the flexibility of this MALDI-MSI approach to address many questions regarding lipid metabolism and function in plants where spatial distribution is an important aspect of biology. These may include studies of developmental processes, tissue-specific/cell type specific metabolism, or cell-cell communication, and the experimental approaches developed here with *Arabidopsis* mutants brings an important new perspective for probing plant lipid function.

One criticism of MALDI-MS imaging approaches has been concerns over differences in the ionization efficiency of metabolites and the resulting erroneous conclusions that can be derived when attempting to understand relative quantities of metabolites *in situ*. Here we have carefully compared the quantities of the acyl-lipid molecular species and found excellent agreement between values obtained with conventional ESI-MS quantification in extracts, and the relative mol% values of phospholipid species summed over all MALDI-MS scans in the embryos (Fig. 5). Therefore, relative quantification of acyl lipid molecular species within a lipid class appears to be quite robust here even in single *A. thaliana* seeds, and the relative quantification of lipid species among tissues shows a marked heterogeneity (Figs. 3, 4, 6-8). The general distributions of molecular species determined here by MALDI-MSI were entirely consistent with reports of fatty acid composition differences between different dissected seed parts in both *A. thaliana* where polyunsaturated 18C fatty acids were enriched in embryonic axis and peripheral endosperm, and saturates and monounsaturates (16:0, 18:1 and 20:1) were

enriched in cotyledons (Li et al, 2006). This consistency in the mol% quantification between MALDI-MSI and molecular species profiles determined from extracts is entirely consistent with our studies elsewhere [17, 18, 21, 33] and those reported by others [34] and demonstrate the high-quality data obtained with MALDI-MSI, especially when interfaced with high resolution mass spectrometers. Nevertheless, it is important that careful validation accompany MALDI-MS images, so that locations of metabolites are accurately reported. It should also be emphasized that the excellent reproducibility of metabolite distributions is evident when data are collected from multiple seeds, or even in multiple, serial sections of the same seed (Figs. 3, 4).

A major advantage of MALDI-MSI is that matrix and instrument conditions can be altered to ionize a wide range of molecules including proteins [35], small molecules[36-38], sugars[39], and numerous other secondary metabolites[40-42]. Consequently, the 3D MALDI-MS imaging approach described here may find broad application for localizing many types of molecules in plant tissues, and support studies in metabolism, signaling and even phytoremediation where the locations of metabolites are of functional importance. Furthermore, by using various imaging processing software packages [43] 3D MALDI-MS reconstructions can be “dissected” virtually, enabling evaluation of metabolite distributions in different tissue types. We anticipate that localization studies of small molecules by MALDI-MSI and other approaches will continue to reveal unexpected heterogeneous distributions, that to date have been largely ignored, but that are a critical feature of biological systems that must be accounted for.

4.2 Implications of Molecular Heterogeneity

Several PC species were distributed differentially in *A. thaliana* seed tissues; e.g., some species were enriched in peripheral endosperm, embryonic axis and/or cotyledonary tissues. While the functional significance of these differences remains to be elucidated, previous studies with seeds of the close *Brassicaceae* relative, *C. sativa*[18], revealed similar heterogeneous distributions for some of the PC species reported here for *A. thaliana*. For example, in both seed types, PC 34:2 and PC 36:4 were enriched in the embryonic axis, whereas PC 38:3 and PC 38:4 were localized preferentially to the cotyledons (Fig. 6A and 7A) [17], suggesting that molecular heterogeneity is likely an evolutionarily conserved process and is necessary for other downstream biological metabolic processes in these oilseeds (e.g. signaling or TAG biogenesis).

The acyl-chains in PC are largely used for the assembly of TAGs in oilseeds through a complex interaction of CoA-dependent and -independent acyltransferases [1] and to some

extent these PC acyl chain distributions in *A. thaliana* seeds may reflect a precursor-product relationship to the acyl chains in TAG. This is supported by MALDI-MSI studies co-localizing PC and TAG molecular species in *C. sativa* seeds [17, 18]. In *C. sativa* seeds, PC 36:4 (18:2/18:2), PC 34:2 (16:0/18:2) and TAG 54:6 (18:2/18:2/18:2) were localized preferentially in the embryonic axis tissues. It was proposed that TAG 54:6 in these tissues may be synthesized through removal of an 18:2 fatty acid moiety by phospholipid:diacylglycerol transferase (PDAT) at the sn-2 position from either PC 36:4 or PC 34:2, and incorporation into the sn-3 position of diacylglycerol (DAG) 36:4 (18:2/18:2) to synthesize TAG 54:6 (18:2/18:2/18:2). Additional evidence that TAG 54:6 may be synthesized through a combination of the Lands cycle and PDAT in these embryonic axis tissues comes from reports that this pathway preferentially incorporates polyunsaturated (e.g. 18:2, 18:3), over monounsaturated or long-chain (e.g. 16:0, 20:1), fatty acids [44]. Most conspicuously, 20C acyl chains were largely excluded from PC in the embryonic axis of *A. thaliana* (Figs. 3, 6A), and instead were enriched in cotyledonary tissues. This cotyledon-enriched distribution in *A. thaliana* was consistent with *C. sativa* seeds, where 20C acyl chains were enriched in PC and TAG in cotyledonary tissues. This prompted speculation that perhaps the diacylglycerol acyltransferase (DGAT) may be preferentially active in cotyledonary tissues for the assembly of TAG, utilizing elongated acyl-CoAs derived from PC for incorporation into TAG [13, 17]. This distinction between PDAT and DGAT pathways for TAG assembly in different seed tissues remains to be demonstrated, but both enzyme activities are known to cooperate in the synthesis of TAG in *A. thaliana* seed tissues [8]. Nonetheless, the distributions of PC molecular species reported here in *A. thaliana* likely reflect their central role in TAG molecular species assembly, and future studies with additional mutants may help reveal tissue-based differences in metabolic pathways.

PI is synthesized in a manner somewhat differently from PC in that the DAG substrate for assembly of the phospholipid class is the activated CDP-DAG. The main PI molecular species in wild-type *A. thaliana* was PI 34:2, but this species was still distributed in a heterogeneous manner consistent with the distribution of PC 34:2, which was more concentrated in the embryonic axis relative to cotyledons (Figs. 4, 8). Although not particularly abundant relative to other PI species (Fig. 8), adjusting the maximum ion counts showed that PI 38:3 was distributed in an opposite manner, more concentrated relatively speaking in cotyledons relative to the embryonic axis (Fig. 4). While the composition of PI is relatively simple and dominated by a single species, its distribution is nevertheless consistent with the major membrane phospholipid (PC), likely a reflection of the 34:2 DAG base contributed following its modification in PC.

4.3 Acyl-Lipid Mutants Demonstrate Molecular Heterogeneity is Driven In Part by Metabolism

Ultimately, the heterogeneous distributions of PC, and PI molecular species appear to be influenced by acyl-modifying enzymes, FAD2, FAD3, FAD6, and FAE1. MALDI-MS images of lipid distributions in mutants of these acyl-modifying enzymes, with the exception of *fad6-1*, demonstrate that they are required to establish the heterogeneous distribution of the molecular species of membrane phospholipids visualized in wild-type (Col-0) seeds (Figs. 6-8). For example, without FAD2 to direct metabolism in either the embryonic axis, cotyledons, or peripheral endosperm, newly-formed predominant molecular species containing 18:1 accumulated homogeneously across the seed. This was the case for molecular species PC 34:1 and PI 34:1 as well as PC 36:2 (Figs. 6B, 7B, and 8B). Interestingly, the molecular species of PC and PI containing polyunsaturated acyl-chains in *fad2-1* seeds were not eliminated, and these residual polyunsaturated species containing 18:2 or 18:3 had a distinct distribution profile of enrichment in the peripheral endosperm and/or in the radicle portion of the embryonic axis (Figs. 6G-8G). This suggests that perhaps additional desaturases may be active in these tissues directing the synthesis of polyunsaturated fatty acids for membrane lipids. Although we cannot rule out that residual FAD2 may be active in this “leaky” mutant, it is curious that this desaturation activity would only be observed in restricted tissues in the embryo and less so in cotyledons.

One possible explanation for the occurrence of polyunsaturated species in the *fad2-1* mutant might be through FAD6, which encodes a prokaryotic pathway desaturase that can desaturate both 16:1 and 18:1 to 16:2 and 18:2 respectively [32]. However, the MALDI-MS images of *fad6-1* did not reveal significant changes spatial distributions when compared to wild-type, perhaps due to FAD2 activity compensating for the loss of FAD6 in the *fad6-1* mutants seeds. Examination of seed gene expression maps for FAD6 revealed that in maturing *A. thaliana* seeds, FAD6 is expressed preferentially in the peripheral endosperm, relative to the rest of the seed [15]. Furthermore, in the *fad3-2* mutants, although differences in heterogeneity were less pronounced, there was a residual PC 34:3 (16:0/18:3, confirmed by MS/MS images and in extracts) that was localized to the peripheral endosperm, again supporting additional desaturases in this tissue, and consequently perhaps a more important, previously unappreciated role for the prokaryotic pathway in assembly of membrane lipids at this location. Hence we favor an explanation whereby FAD6 is contributing to the phospholipid heterogeneity

in *fad2-1* seeds, especially for the molecular species containing 18:2 or 18:3; further experiments, such as with higher order mutants, will be required to sort out this interesting phenomenon. In any case, this demonstrates the value of combining lipid imaging to localize lipid metabolites *in situ* with genetic mutants to glean new information about the complexities of lipid metabolism in plant tissues.

As might be expected, FAE1 appears to be required for establishing heterogeneity of the long-chain 38C molecular species of PC, as it encodes a 3-ketoacyl-CoA synthase that is the first step in fatty acid elongation (e.g. 18:1-CoA to 20:1-CoA) in the cytosol. In wild type seeds, 38C PC molecular species are primarily enriched in the cotyledons and hypocotyls and are nearly absent in the radicle (Fig. 6A). Analysis of *fae1-1* seeds by MALDI-MS imaging *in situ* (and in extracts) confirmed that 38C PC molecular species were completely absent (Fig. 6E-G). Rossak et al. investigated the expression patterns of FAE1 gene in *A. thaliana* seeds and demonstrated by both *insitu* hybridization and promoter-GUS fusions that *FAE1* is expressed exclusively in cotyledons and hypocotyl tissues and was essentially absent from the radicle tip [45]. Here, the MALDI-MS images of the PC molecular species containing 20:1 acyl groups, PC 38:3 and PC 38:4 (Fig. 6A) match exactly the expression patterns of *FAE1*, and it is simply the tissue specific expression of *FAE1* that is responsible for both composition and location of elongated fatty acids in PC. Hence, heterogeneity observed for PC in the 38C moieties then is likely a reflection of the high demand of these fatty acids for the assembly of seed storage lipids in cotyledonary and hypocotyl tissues, and 38C PC species serve primarily as intermediates in the formation of DAG for TAG synthesis.

4.4 Future Considerations for 3D MALDI-MS Imaging

There are several technical challenges for the wide-use of 3D MALDI-MSI. First, it is not trivial to reproducibly acquire tens of consecutive MALDI-MS data sets. This task is especially challenging when the tissue size is very large and/or when each data set takes several hours to acquire. Second, it is difficult to perfectly align multiple 2D MALDI-MSI data sets into the third axis. Currently, we rely on manual alignment of each 2D MSI image with the corresponding optical image, which is laborious and subject to potential error. Last, the current 3D MALDI-MS imaging reconstructions are done for individual *m/z*s, one at a time; for each *m/z*, 2D MALDI-MS images are created for each data set, followed by alignment, and 3D rendering. Some of these limitations are expected to be able to overcome in the future, especially with the development of automatic computational tools and improved MALDI-MS instrumentation.

4.5 Conclusions

Here we have presented the first report of three-dimensional MALDI-MSI reconstruction of plant tissues, and have utilized this analytical technique to visualize several PC and PI molecular species in 3D in seeds of *Arabidopsis thaliana*. These 3D MALDI-MSI reconstructions were used to quantify in a relative manner, the major and minor PC and PI molecular species in the individual tissue types of the cotyledons, embryonic axis, and peripheral endosperm, which combined, matched with excellent agreement with bulk extracted lipids quantified by ESI-MS. Using 2D MALDI-MSI the lipid distributions of PC and PI in *A. thaliana* seeds were described in wild type, *fad2-1*, *fad3-2*, *fad6-1*, and *fae1-1* acyl lipid mutants revealing previously unappreciated spatial distributions of membrane phospholipids and likely will be used to further understand the spatial regulation of these genes. Future studies with additional mutants and/or other lipid species, especially during development, may help to uncover additional temporal and spatial processes required for proper compartmentalization of seed development.

Fig. Legends:

Fig. 1: Workflow for MALDI-MSI Three-Dimension Reconstruction of Lipid Localization(A)

Scanning electron micrograph of a wild type *A. thaliana* seed, scale bar represents 100 μm . (B)

Stacked projection of serial longitudinal cryo-sections of an entire *A. thaliana* seed. (C)

Illustration of two-dimension MALDI-MSI data acquisition where a laser is rastered over a selected area of tissue and ions are generated and recorded at every x and y position, which can then be queried for a selected m/z ion with high mass resolution to generate two-dimension MALDI-MS images. (D) Stacked projection and alignment of serial two-dimension MALDI-MS images acquired from their respective cryo-sections from the *A. thaliana* seed. (E) Three-dimension MALDI-MS image reconstruction of an *A. thaliana* seed from two-dimensional high resolution MALDI-MS images.

Fig. 2: Anatomy of *A. thaliana* Seeds and Optical Images of Serial Cryo-Sections.(A-B)

Bright-field images of sections of medial, longitudinal and cross-sectional orientations of *A. thaliana* seeds with the embryonic axis, peripheral endosperm, cotyledons, and seed coat labeled. (C-D) Bright-field images of serial longitudinal and transverse cryo-sections of entire *A. thaliana* seeds. The order of the sections is notated at the top left hand corner of each image. All images for both the longitudinal and transverse sections are oriented where the embryonic axis is localized on top and cotyledons at the bottom of the images. Scale bars represent 100 μm .

Fig. 3: Two-Dimension MALDI-MS images and Three-Dimension Reconstructed Images for Phosphatidylcholine Molecular Species.

Serial two-dimension MALDI-MS images of (A) embryonic-axis-enriched PC 34:2 m/z 758.571, (B) peripheral-endosperm-enriched PC 34:3 m/z 756.554, and (C) cotyledon-enriched PC 38:3 m/z 812.614 molecular species. Three-dimensional reconstructed images are shown at the bottom (D-F) by overlaying all 2D images. All PC species were detected as protonated, $[M+H]^+$. Ion intensities are shown using a blue to red scale, with red representing the highest intensity. Images were generated by normalizing each ion signal to total ion count at each voxel. Scale bars represent 200 μm .

Fig. 4: Two-Dimension MALDI-MS images and Three-Dimension Reconstructed Images for Phosphatidylinositol Molecular Species.

Serial two-dimension MALDI-MS images of (A) embryonic-axis-enriched PI 34:2 at m/z 833.518 and (B) cotyledon-enriched PI 36:3 at m/z 859.533 molecular species. Three-dimensional reconstructed images are shown at the bottom (C-D) by overlaying all 2D images. All PI species were detected as deprotonated, $[M-H]^-$. Ion

intensities are shown using a blue to red scale, with red representing the highest intensity. Images were normalized to total ion count. Scale bars represents 100 μm .

Fig. 5: Three-Dimension MALDI-MSI mol% Profiles of Phosphatidylcholine and Phosphatidylinositol Molecular Species for Single Seed and Individual Tissue Types. (A)

The PC ion intensities, **(B)** the optical images, and **(C)** the respective PC mol% profiles for selected representative sections. The PC mol% profiles from each section were calculated by “scaling” to the number of voxels of tissue. **(D)** Comparison of the 3D PC mol% profiles between “scaling” of 2D PC mol% with each occupying voxels and normalization of sum of unadjusted ion intensities. **(E)** Clustered column, representing sum of the 3D PC mol% profiles of each embryonic axis, cotyledons and peripheral endosperm as a part of the single seed 3D mol% profile, compared to the PC mol% profile obtained from the total lipid extract analyzed by direct infusion ESI-MS. **(F)** Clustered column, representing the 3D PI mol% profile of each embryonic axis and cotyledons as a part of the single seed 3D mol% profile, compared to the PI mol% profile obtained from the total lipid extract analyzed by direct infusion ESI-MS.

Fig. 6: Two-Dimension MALDI-MS Imaging of Phosphatidylcholine 34C and 38C

Molecular Species in *A. thaliana* Acyl-Lipid Mutants. (A-E) Two-dimension MALDI-MS images of medial longitudinal sections mapping the spatial distributions of PC 34C and 38C molecular species in wild type, *fad2-1*, *fad3-2*, *fad6-1*, and *fae1-1A. thaliana* seed acyl-lipid mutants, respectively. All PC molecular species were detected as $[M+H]^+$ ions and ion signals were normalized to total ion count. Assignments were made based on accurate mass values and confirmed by direct infusion ESI-MS precursor and product scan analysis. Ion intensities are shown using a blue to red scale, with red representing the highest intensity, and intensities of MS images has been set to the same intensity respective of genotype. Scale bars represents 100 μm . Distributions were determined in at least three biological replicates. **(F)** Average mol% of 34C and 38C PC molecular species obtained from the MALDI-MS images of medial longitudinal seed sections ($n=3$ from three individual seed sections) **(G)** Average mol% of 34C and 38C PC molecular species obtained from the analysis of total lipid extracts analyzed by direct infusion ESI-MS detecting for the precursor ion of the PC head group m/z 184 ($n=3$ from ~250 seeds).

Fig. 7: Two-Dimension MALDI-MS Imaging of Phosphatidylcholine 36C Molecular Species

in *A. thaliana* Acyl-Lipid Mutants. (A-E) Two-dimension MALDI-MS images of medial longitudinal sections mapping the spatial distributions of PC 36C molecular species in wild type, *fad2-1*, *fad3-2*, *fad6-1*, and *fae1-1A. thaliana* seed acyl-lipid mutants, respectively. All PC

molecular species were detected as $[M+H]^+$ ions and ion signals were normalized to total ion count. Assignments were made based on accurate mass values and confirmed by direct infusion ESI-MS precursor and product scan analysis. Ion intensities are shown using a blue to red scale, with red representing the highest intensity, and intensities of MS images has been set to the same intensity respective of genotype. Scale bars represents 100 μm . Distributions were determined in at least three biological replicates. **(F)** Average mol% of 36C PC molecular species obtained from the MALDI-MS images of medial longitudinal seed sections (n=3 from three individual seeds). **(G)** Average mol% of 36C PC molecular species obtained from the analysis of total lipid extracts analyzed by direct infusion ESI-MS detecting for the precursor ion of the PC head group m/z 184 (n=3 from ~250 seeds).

Fig. 8: Two-Dimension MALDI-MS Imaging of Phosphatidylinositol Molecular Species in A. thaliana Acyl-Lipid Mutants. (A-E) Two-dimension MALDI-MS images of medial transverse sections mapping the spatial distributions of major PI molecular species in wild type, *fad2-1*, *fad3-2*, *fad6-1*, and *fae1-1A. thaliana* seed acyl-lipid mutants, respectively. All PI molecular species were detected as $[M-H]^-$ ions and ion signals were normalized to total ion count. Assignments were made based on accurate mass values and confirmed by direct infusion ESI-MS neutral loss and product scan analysis. Ion intensities are shown using a blue to red scale, with red representing the highest intensity, and intensities of MS images has been set to the same intensity respective of genotype. Scale bars represents 100 μm . Distributions were determined in at least three biological replicates. **(F)** Average mol% of PI molecular species obtained from the MALDI-MS images of medial transverse seed sections (n=3 from three individual seed sections) **(G)** Average mol% of PI molecular species obtained from the analysis of total lipid extracts analyzed by direct infusion ESI-MS detecting for the neutral loss of the PI head group m/z 277 (n=3 from ~250 seeds).

Table 1: ESI-MS Product Ion Analysis and Acyl-Chain Designation of Phosphatidylcholine and Phosphatidylinositol Lipid Molecular Species from Total Lipid Extracts. Acyl chain fragments were identified from spectra of product ion scans from their respective parent ion. PC parent ions were detected as $[M+OAc]^-$, PI ions were detected as $[M+H]^-$ [24,25].

Supplementary Video 1: Three-Dimension Video of MALDI-MS Reconstruction Mapping Embryonic axis Enriched PC 34:2.

Supplementary Video 2:Three-Dimension Video of MALDI-MS Reconstruction Mapping Peripheral Endosperm Enriched PC 34:3.

Supplementary Video 3:Three-Dimension Video of MALDI-MS Reconstruction Mapping Cotyledon Enriched PC 38:3.

Supplementary Video 4:Three-Dimension Video of MALDI-MS Reconstruction Mapping Enriched PI 34:2.

Supplementary Video 5:Three-Dimension Video of MALDI-MS Reconstruction Mapping Cotyledon Enriched PI 36:3.

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Table 1: ESI-MS Product Ion Analysis and Acyl-Chain Designation of Phosphatidylcholine and Phosphatidylinositol Lipid Molecular Species from Total Lipid Extracts. Acyl chain fragments were identified from spectra of product ion scans from their respective parent ion. PC parent ions were detected as [M+OAc]⁻, PI ions were detected as [M+H]⁺.

Lipid Molecular Species	Chemical Formula	Mass Used for Product Ion Analysis	Wild Type	fad2-1	fad3-2	fad6-1	fae1-1
<i>m/z of [M+OAc]⁻</i>							
PC-34:3	C ₄₂ H ₇₈ O ₈ PN	814	18:3-16:0	18:2-16:1, 18:3-16:0	18:2-16:1>18:3-16:0	18:2-16:1, 18:3-16:0	18:3-16:0, 18:2-16:1
PC-34:2	C ₄₂ H ₈₀ O ₈ PN	816	18:2-16:0	18:1-16:1>18:2-16:0	18:2-16:0>18:1-16:1	18:2-16:0	18:2-16:0>18:1-16:1
PC-34:1	C ₄₂ H ₈₂ O ₈ PN	818	18:1/16:0	18:1-16:0	18:1-16:0	18:1-16:0	18:1-16:0
PC-36:6	C ₄₄ H ₇₆ O ₈ PN	836	18:3-18:3	18:3-18:3	18:3-18:3	18:3-18:3	18:3-18:3
PC-36:5	C ₄₄ H ₇₈ O ₈ PN	838	18:3-18:2	18:3-18:2	18:3-18:2	18:3-18:2	18:3-18:2
PC-36:4	C ₄₄ H ₈₀ O ₈ PN	840	18:2-18:2, 18:3-18:1	18:3-18:1>18:2-18:2	18:2-18:2>>18:3-18:1	18:2-18:2>>18:3-18:1	18:2-18:2>18:3-18:1
PC-36:3	C ₄₄ H ₈₂ O ₈ PN	842	18:2-18:1	18:2-18:1>18:3-18:0	18:2-18:1	18:2-18:1	18:2-18:1>18:3-18:0
PC-36:2	C ₄₄ H ₈₄ O ₈ PN	844	18:1-18:1	18:1-18:1>>18:2-18:1	18:1-18:1>18:2-18:0	18:1-18:1>18:2-18:0	18:1-18:1>18:2-18:0
PC-36:1	C ₄₄ H ₈₆ O ₈ PN	846	18:1-18:0>20:1-16:0	18:1-18:0>20:1-16:0	18:1-18:0>20:1-16:0	18:1-18:0>20:1-16:0	18:1-18:0
PC-38:4	C ₄₆ H ₈₄ O ₈ PN	868	20:1-18:3, 20:2-18:2	20:1-18:3	20:2-18:2>20:1-18:3	20:1-18:3, 20:2-18:2	20:2-18:2>20:1-18:3
PC-38:3	C ₄₆ H ₈₆ O ₈ PN	870	20:1-18:2	20:2-18:1, 20:1-18:2	20:1-18:2, 20:2-18:1	20:1-18:2	20:1-18:2
PC-38:2	C ₄₆ H ₈₈ O ₈ PN	872	20:0-18:2, 20:1-18:1	20:1-18:1	20:0-18:2, 20:1-18:1	20:1-18:1, 20:0-18:2	20:0-18:2
<i>m/z of [M-H]⁺</i>							
PI-34:3	C ₄₃ H ₇₇ O ₁₃ P	831	18:3-16:0	18:3-16:0	18:3-16:0, 18:2-16:1	18:3-16:0	18:3-16:0>>18:2-16:1
PI-34:2	C ₄₃ H ₇₉ O ₁₃ P	833	18:2-16:0	18:2-16:0>>18:1-16:1	18:2-16:0	18:2-16:0	18:2-16:0
PI-34:1	C ₄₃ H ₈₀ O ₁₃ P	835	18:1-16:0	18:1-16:0	18:1-16:0	18:1-16:0	18:1-16:0
PI-36:4	C ₄₅ H ₇₉ O ₁₃ P	855	18:2-18:2>18:3-18:1	18:3-18:1>>18:2-18:2	18:2-18:2	18:2-18:2>>18:3-18:1	18:2-18:2>18:3-18:1
PI-36:3	C ₄₅ H ₈₁ O ₁₃ P	857	18:2-18:1>18:3-18:0	18:2-18:1>>18:3-18:0	18:2-18:1	18:2-18:1	18:2-18:1>18:3-18:0
PI-36:2	C ₄₅ H ₈₃ O ₁₃ P	859	18:2-18:0>18:1-18:1	18:1-18:1>>18:2-18:0	18:2-18:0	18:2-18:0>18:1-18:1	18:2-18:0>18:1-18:1

Figure 1

Figure 2

Figure 3

Figure 4

A PI 34:2 m/z 833.518

B PI 36:3 m/z 859.533

C PI 34:2 m/z 833.518

D PI 36:3 m/z 859.533

MIN MAX

Figure 5

Figure 6

Figure 7

Figure 8

A Wild Type (Col-0)

B fad2

C fad3

D fad6

E fae1

Bright-Field PI 34:3 PI 34:2 PI 34:1 PI 36:4 PI 36:3 PI 36:2
(*m/z* 831.503) (*m/z* 833.518) (*m/z* 835.534) (*m/z* 857.518) (*m/z* 859.534) (*m/z* 861.549)

F

G