TECHNICAL ADVANCE

High-Spatial Resolution Mass Spectrometry Imaging reveals the genetically programmed, developmental modification of the distribution of thylakoid membrane lipids among individual cells of the maize leaf

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SUMMARY

Metabolism in plants is compartmentalized among different tissues, cells and subcellular organelles. Mass spectrometry imaging (MSI) with matrix-assisted laser desorption ionization (MALDI) has recently advanced to allow for the visualization of metabolites at single cell resolution. Here we applied 5 and 10 μm high-spatial resolution MALDI-MSI to the asymmetric Kranz anatomy of maize leaves to study the differential localization of two major anionic lipids in thylakoid membranes, sulfoquinovosyldiacylglycerols (SQDG) and phosphatidylglycerols (PG). The quantification and localization of SQDG and PG molecular species, among mesophyll (M) and bundle sheath (BS) cells, are compared across the leaf developmental gradient from four maize genotypes (the inbreds B73 and Mo17, and reciprocal hybrids B73xMo17 and Mo17xB73). SQDG species are uniformly distributed in both photosynthetic cell types regardless of leaf development or genotype. However, PG shows photosynthetic cell-specific differential localization depending on the genotype and the fatty acyl chain constituent. Overall, 16:1-containing PGs primarily contribute to the thylakoid membranes of M cells while BS chloroplasts are mostly composed of 16:0-containing PGs. Furthermore, PG 32:0 shows genotype-specific differences in cellular distribution, with preferential localization in BS cells for B73, but more uniform distribution between BS and M cells in Mo17. Maternal inheritance is exhibited within the hybrids such that localization of PG 32:0 in B73xMo17 is similar to the distribution in the B73 parental inbred, whereas that of Mo17xB73 resembles the Mo17 parent. This study demonstrates the power of MALDI-MSI to reveal unprecedented insights on metabolic outcomes in multicellular organisms at single cell resolution.

Significance Statement

High-spatial resolution mass spectrometry imaging is applied to study the differential localization of sulfoquinovosyldiacylglycerols (SQDG) and phosphatidylglycerols (PG) in thylakoid membranes of maize leaves. SQDG shows homogeneous distributions in bundle sheath and mesophyll cells, regardless of genotype or developmental stage; however, PG shows fatty acyl chain dependent non-uniform distributions, influenced by genotype and development.

INTRODUCTION

Thylakoid membranes play an important organizational role in maintaining the structural integrity of photosystem complexes (Quinn and Williams 1983). The thylakoid membrane is the universal site of the photochemical and electron transport reactions of oxygenic photosynthesis in cyanobacteria and plant chloroplasts. The major lipids within the thylakoid membrane are the neutral galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which together account for approximately 80% of total thylakoid lipids in plant chloroplasts (Kobayashi *et al.* 2009). The remaining thylakoid membrane lipids are mainly composed of anionic lipids sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Demé *et al.* 2014, Sakurai *et al.* 2006); these negatively charged lipids have been shown to be responsible for the structural and/or functional integrity of photosystems I and/or II (Guskov *et al.* 2009, Jordan *et al.* 2001, Sato 2004, Umena *et al.* 2011).

A number of studies have highlighted that there are significant differences in the fatty acyl chains associated with different thylakoid lipid molecular species (Hsu *et al.* 2007). These composition patterns can be modified by both the genetic and developmental programs of the organism, and by environmental stimuli that the organism experiences (Ben Hamed *et al.* 2005, Pál *et al.* 2007, Xu *et al.* 2003). Galactolipids (MGDG and DGDG) are characterized by an exceptionally high trienoic fatty acid content, mainly α-linolenic acid (Duchêne and Siegenthaler 2000, Nishihara *et al.* 1980), while the major lipid species in SQDG are enriched in palmitic acid. PG is a distinct thylakoid lipid, in that its acyl composition is vastly different depending on the plant species.

Unlike C3 plants, maize employs a highly efficient C4-type photosynthesis that is differentially regulated by cellular compartmentalization of photosynthetic carbon assimilation in

the mesophyll (M) cells, and photosynthetic carbon reduction in the bundle sheath (BS) cells, creating a two-cell metabolism system. In addition to the metabolic flux that occurs between these two cell types, ultrastructural and photosystem assays have revealed that the complexity of C4 photosynthesis is also dependent on the composition differences of the chloroplast envelopes and thylakoid membranes (Manandhar-Shrestha *et al.* 2013). One of these differences is associated with the acyl-chain composition of PG, which appears to be different in the thylakoid membranes of M and BS cells. Specifically, PG with a 16:1 fatty acid at the sn-2 position (e.g., PG 16:0/16:1) is highly enriched in M chloroplasts, and PG with 16:0 at the sn-2 position (e.g., PG 16:0/16:0) is primarily localized in BS chloroplasts (Nishihara, et al. 1980). Moreover, the acyl-chains of PG are correlated with developmental stages as well as chilling sensitivity. For example, plants with high levels of disaturated thylakoid PGs are shown to be chilling-sensitive (Roughan 1985); however, this is not without debate (Kaniuga *et al.* 1999).

In plants, grass species serve as an excellent model to study the establishment of various functions relative to the developmental gradient of leaves, in which the youngest cells are situated at the base of the leaf, and the oldest and most mature cells at the leaf tip (Nelson and Langdale 1992). Cellular and photosynthetic differentiation occurs in a controlled manner in a basipetal, or tip-to-base, fashion (Evert *et al.* 1996, Kirchanski 1975, Leech *et al.* 1973) with proplastids present at the base of the leaf blade and fully differentiated BS and M chloroplasts at the tip (Evert, et al. 1996, Majeran and van Wijk 2009, Nelson and Langdale 1992). A number of studies have characterized the transcriptomic and metabolomic changes across the developmental gradient of maize leaves (Li *et al.* 2010, Pick *et al.* 2011, Wang *et al.* 2014). Differential transcriptome (Sharpe *et al.* 2011, Tausta *et al.* 2014) and proteome (Majeran *et al.* 2005, Majeran *et al.* 2008, Manandhar-Shrestha, et al. 2013) expression have been identified between

M and BS cells. However, because these omics studies were performed through extraction of ground tissue, *in situ* localization for metabolites or proteins, for example, were not preserved.

Mass spectrometry imaging (MSI) has become a valuable tool for analyzing spatial distributions of a wide range of compounds directly on or within plant and animal tissues (Angel and Caprioli 2013, Jungmann and Heeren 2012, Lee *et al.* 2012, Sturtevant *et al.* 2016, Svatos 2010, van Hove *et al.* 2010, Zaima *et al.* 2010). Different ionization techniques have been adopted for MSI; however, matrix assisted laser desorption ionization (MALDI) is most attractive in terms of high spatial resolution, sensitivity, and chemical versatility, which is essential for *in situ* single cell resolution imaging. The spatial resolution of MALDI-MSI has become routinely available in the range of 20-30 μm, and has been demonstrated to as high as 2.5 μm resolution (Zavalin *et al.* 2015).

Recently, we have established a MALDI-MSI technical platform with a MALDI-linear ion trap Orbitrap mass spectrometer for 5-10 μm high-spatial resolution and demonstrated the visualization of the distribution of a number of different metabolites in cross-sections of maize leaves (Korte *et al.* 2015). Cell-type specific non-uniform distribution of PG, previously indicated by bulk chloroplast analysis from physically separated BS and M cells (Nishihara, et al. 1980), was confirmed using this single cell level direct *in situ* MSI analysis platform (Korte, et al. 2015). In the previous work, we have achieved 5 μm spatial resolution using oversampling method with 9 μm laser spot size. In the present work, we used an improved laser spot size of 6~7 μm with minimal oversampling which allows clearer image especially for small size cell, and applied the high-resolution MALDI-MSI platform to explore quantitative fatty acyl distributions of PG and SQDG along the developmental gradient of maize leaves of two inbred lines, B73 and Mo17, and the reciprocal hybrid lines, B73xMo17 and Mo17xB73.

RESULTS

Morphology and Lipid Distribution during Leaf Development

Fatty acid composition of thylakoid membrane lipids was studied at four consecutive developmental zones in the maize seedling leaf: the basal zone, maturing proximal zone, maturing midpoint zone, and mature distal zone as defined by Li and colleagues (Li, et al. 2010). The overall workflow is shown in Figure S1 and described in detail in the Experimental Procedures section. The third true leaf was harvested from seedlings of inbreds B73 and Mo17, and reciprocal hybrids B73xMo17, and Mo17xB73 11-13 days after imbibition, at a seedling height of 14-18 cm. Each sampled leaf was immediately flash frozen, and cryo-sectioned at four specific positions along the developmental gradient of the leaf, as illustrated in Figure S2: 1) Basal Zone, ~1.5 cm below the ligule of leaf 2; 2) Proximal, ~1.5 cm above the ligule of leaf 2; 3) Midpoint, halfway between proximal end and the leaf tip; and 4) Distal, ~2 cm from the leaf tip.

High resolution optical images and anatomical assignments are shown in Figure S3 for the distal and proximal sections of a B73 leaf. In the optical images of Figures 1 and 2, both inbred lines and the two hybrid lines exhibit the characteristic C4 Kranz anatomy (Brown 1975, Haberlandt 1882), in which concentric rings of BS cells encircle closely spaced veins [vascular bundles (VB)], and are surrounded by M cells. Chloroplasts are mostly located along the boundary of M and BS cells. At the basal zone of the third leaf, Kranz anatomy can be distinguished but is not fully differentiated and few mature chloroplasts are observed (Kirchanski 1975). Because growth rates differ among the four genotypes, leaves from the B73 inbred and the B73xMo17 hybrid were harvested one to two days prior to the Mo17 inbred and Mo17xB73

hybrid, such that leaves from each genotype were of similar lengths. Nevertheless, as revealed by the optical images, the sizes of the cells are smaller in inbred Mo17 and hybrid Mo17xB73.

Supplementary figures (Figures S4-S7) show the MS images for all the molecular species of PG and SQDG obtained at 10 μm spatial resolution, as well as several additional metabolites that are associated with photosynthesis, i.e., chlorophyll *a*, plastoquinone/plastoquinol, and carotene. Assignments are based on accurate mass measurements, and their identities are also confirmed by separate MS/MS measurements for the lipids (Figures S8-S9). The presence and cellular distribution of these metabolites and thylakoid membrane lipids were similar in replicate imaging experiments conducted on three independent seedlings. Figures 1 and 2 present the representative images of three thylakoid membrane lipids (PG 32:0, PG 32:1, SQDG 34:3) and chlorophyll *a*, comparing between the inbreds and the reciprocal hybrids, respectively.

Consistent with the photosynthetic developmental gradient that is visualized by the progression from etiolation at the base to green pigmentation at the tip of the seedling leaf, the ion signal intensity for chlorophyll *a* increases from the base of the leaf to the tip. MS imaging visualization of the cross-sectional distribution of chlorophyll *a* indicates that it is highly abundant in BS cells (Figures 1 and 2). Higher magnification MS images indicate the occurrence of chlorophyll *a* in M cells (Figures S10 and S11), but it occurs at much lower abundance than in BS cells. In contrast to chlorophyll *a* that can absorb UV at 355 nm, which is the wavelength of the laser used for these experiments and can thus be ionized even without a matrix, chlorophyll *b* does not absorb at this wavelength, and could not be detected in these experiments. The fact that chlorophyll *a* is highly concentrated in the thylakoid membranes of BS cells can be used to guide the localization of SQDGs and PGs between BS and M cells (Kirchhoff *et al.* 2013). Chlorophyll fluorescence image obtained for a consecutive tissue section agrees well with the MS image,

validating our technology (Figure S12). It needs to be noted that chlorophyll *a* may act as a matrix itself due to its laser absorption, but its effect is expected to be minimum compared to DAN matrix according to our experiment on standard samples (Figure S13).

SQDG 34:3 distribution is homogeneous between both the photosynthetic cell types, regardless of genotype and stage of development (Figure 1M-P and 1M'-P', and Figure 2M-P and 2M'-P'). In contrast, PGs are differentially distributed among M and BS cells depending on the molecular species, and this non-uniform distribution is affected by genotype, being different among the two inbreds and the hybrids. As we previously reported for the midpoint section (Korte, et al. 2015), in inbred B73, PG 32:0 is primarily located in BS cells (Figure 1E-H) and PG 32:1 is almost exclusively present in M cells (Figure 1I-L); this distribution is unaffected by leaf development, but the MS images are clear only for the midpoint and distal sections and absent or unclear at the basal zone and the proximal end. In Mo17 however, PG 32:1 is not observed in the immature midpoint section as well as the basal and proximal zones, and accumulates to low levels in M cells only in the distal section (Figure 1I'-L'). In contrast to B73, PG 32:0 in Mo17 has a broader distribution (Figure 1E'-1H') occurring in both BS and M cells and this distribution is not affected by development. This broader distribution of PG32:0 in Mo17 across developmental stages is very similar to the observed distribution for SQDG 34:3 (Figures 1M'-1P'). In the hybrids, the distribution of these lipids generally mirrors the maternal inheritance pattern, with B73xMo17 showing similar patterns with that of the female parent, B73, and in the Mo17xB73 hybrid the pattern is similar to the female parent, Mo17 **(**Figure 2). Specifically, PG 32:0 is located mostly in BS cells for B73xMo17 and in both M and BS cells for Mo17xB73. These distribution patterns are more evident at 5 μm spatial resolution, especially when overlaid with the optical images (Figures 4, S10, and S11).

Relative Quantification of Lipids

Relative quantification of each SQDG and PG lipid molecular species was determined by averaging the mass spectra in the MALDI-MSI dataset, and calculating the relative abundance by normalizing to the total ion signal of each lipid class. This relative quantification normalized within the same class has been shown to be a reliable approach to compare different fatty acyl chains within the same lipid class (Horn *et al.* 2012). To further evaluate the analytical variation of our approach, we performed MALDI MSI analysis of five consecutive sections of maize leaf and compared their relative quantification of SQDG and PG species (Figure S14). As shown in this data, the deviation between consecutive sections is minimal which suggests that the analytical variation in this analysis is ignorable compared to biological variations. In electrospray ionization, it has been known that the ionization efficiency of lipids is predominantly affected by polar head groups and the length of acyl chain and the degree of saturation is only minimally affect the ionization (Han and Gross 1994, Han *et al.* 2012). To demonstrate it is also true in MALDI-MS, we compared MALDI-MS and ESI-MS of total lipid extract of maize leaf. As shown Figure S15, they showed similar trend except for some minor difference in PG 34:4.

Figure 3 shows the relative abundance of the individual molecular species of SQDG and PG at the four developmental positions of the leaf. SQDG composition is similar across genotypes at each of the leaf positions that are exposed to light (i.e., the proximal, midpoint and distal positions), with SQDG 34:3 being the predominant species (approx. 60-70 mol% in each genotype), SQDG 32:0 comprising 10-20 mol%, and SQDG 34:2 and 36:6 each comprising 5-15 mol%. In the etiolated tissue of the basal zone, however, SQDG 34:2 is the primary thylakoid lipid and comprises 40 mol%, which is significantly higher than any of the exposed portions of

the leaf which contain 5-15 mol% ($p < 0.001$ for all genotypes). SQDG 34:3 is no longer the most dominant species in the basal zone and the relative abundance is significantly less than the leaf zones exposed to light ($p < 0.001$ for all genotypes). Also notable is the fact that there is no evidence for the presence of palmitoleate (16:1 fatty acyl) containing SQDG species, such as SQDG 32:1 or 34:1. This suggests that the observed SQDG species are composed of 16:0 and 18:x fatty acids (FAs) (x=0-3). FA 18:2 is most abundant in the basal zone of the leaf (i.e., SQDG 34:2), but is rapidly replaced by FA 18:3 (i.e., SQDG 34:3) as the leaf develops from proximal to distal portions. These observations are supported by complementary MS/MS experiments (Figure S8) and are in agreement with the previous characterization of thylakoid lipids from isolated BS and M cells (Nishihara, et al. 1980). There are some statistically supported genotypic differences in the relative abundances of these species but these are mostly minor differences.

The fatty acid compositional changes in PG are similar to SQDG, in that 18:2-containing PG is abundant at the basal zone (i.e., high abundance of PG 34:2) but it is gradually replaced by 18:3-containing PG species, correlated with the emergence of the seedling leaf into direct illumination (i.e., increase of PG 34:3 and decrease of PG 34:2). However, accumulation patterns of PG species are more dynamic across the developmental gradient compared to SQDG species. Specifically, the decrease of 34:2 and the increase of 34:3 species are more gradual in PG as development occurs, whereas there are only minimal changes in SQDG for the leaf sections exposed to light. In PG lipids, PG 34:3 is present at trace levels in the basal zone and increases up to 20-40 mol% as the leaf develops, but is not the most predominant species in any of the developmental stages. In contrast, the analogous SQDG 34:3 is the predominant species of

SQDG (~60 mol%) in all leaf zones with the exception of the etiolated basal zone, in which it comprises only ~20-40 mol%.

PG composition also differs from SQDG composition relative to FA 16:1 and FA 16:0-containing species. Low but distinct amounts of FA 16:1-containing PG species (i.e., PG 32:1, PG 34:1 and PG 34:4) accumulate as the leaf develops, whereas FA 16:1-containing species are completely absent in SQDG. This is also consistent with MS/MS analysis, which revealed the existence of two species of PG 34:3, 18:3/16:0 and 18:2/16:1 (Figure S9C), whereas SQDG 34:3 is comprised of only 18:3/16:0 (Figure S8B). PG species containing two FA 16:0 (i.e., PG 32:0) accumulate at very low levels in the basal zone and increases to 20-35 mol% in the distal sections, in contrast to SQDG 32:0 which has no or minimal change in the relative abundance across the developmental gradient ($p > 0.05$ for most pair-wise comparison). In addition, genotypic differences in PG 32:0 accumulation are observed in the proximal sections when comparing the inbred parents to the reciprocal hybrids ($p<0.05$), which gradually disappears in later developmental zones, with almost no difference at the distal section. The major contrasts in FA 16:0 and FA 16:1 compositions between PG and SQDG species suggest that such changes may be correlated to the maturity of photosynthetic cells. It is important to note that PG 36:x species were not detected in this study. They are observed in both ESI-MS and MALDI-MS analysis of the total extract but in very low abundance, <0.5% (Figure S15). It is attributed to the high detection limit of high-resolution MS imaging experiment because of the limited sampling volume.

Analysis of Cellular Distributions via High-resolution Mass Spectrometry Images

Close examination of optical microscopic images from leaf cross sections in Figures 1 and 2 indicates distinct genetic-based size differences between M and BS cells. Specifically, inbred Mo17 and hybrid Mo17xB73 BS cells are 10-20 μm in diameter, whereas BS cells in inbred B73 and hybrid B73xMo17 are twice as large with 20-40 μm in diameter. Because of the small BS cell sizes, 10-μm resolution used in Figures 1 and 2 is not sufficient to clearly distinguish cellular distribution of some lipids in Mo17 and Mo17xB73. To clarify cell-type specific localization of PG species, an MS imaging experiment was performed at 5 μm resolution for the midpoint and distal sections. The MS images of five PG species are compared side-by-side between each genotype and between the two developmental stages (Figure 4). For better visualization of the cellular localization of these lipids, the generated MS images are overlaid and aligned with optical microscope images obtained in parallel from consecutive leaf cross-sections. The MS images of PG species are also compared with those of SQDG 34:3 and chlorophyll *a* as shown in Figures S10 and S11 for the midpoint and distal sections, respectively.

For inbred B73 and hybrid B73xMo17, PG 32:1 and PG 34:4 are primarily localized in M cells in both the midpoint and distal sections zones (Figures 4E-F, 4M-N), whereas the other three PGs (PG 32:0, 34:3 and 34:2) are accumulated at low levels in M cells and at much higher levels in BS cells (Figures 4I-J, 4Q-R, 4U-V). Consistent with the mol% distribution of PG 32:1 data shown in Figure 3B', PG 32:1 is almost absent in Mo17 and Mo17xB73 images at the midpoint (Figures 4G-H), and appears to be substituted by PG 32:0, which is present in both M and BS cells (Figures 4K-L). PG 32:1 is clearly visible at the distal section for Mo17xB73 (Figure 4G) and slightly for Mo17 (Figures 4H), and localized mostly in M cells, like B73 and B73xMo17 (Figures 4E-F). PG 32:0, however, is still present in both BS and M cells for Mo17 and Mo17xB73 (Figures 4K-L). For both midpoint and distal sections, the PG 34:2 (Figures 4U-

X) and PG 34:3 (Figures 4Q-T) species show similar localization patterns across all genotypes, in that both species are concentrated in BS cells. The two species that constitute PG 34:3, namely 18:2/16:1 and 18:3/16:0, cannot be distinguished here. PG 34:4 (Figures 4M-P) shows localization concentrated in M cells; this localization pattern is similar across all genotypes in both midpoint and distal sections. Similar distributions between midpoint and distal sections for these PG species further confirms differential localization is not changing as development occurs.

MS/MS imaging of PG 34:3 molecular species

Because the two structural isomers of PG 34:3 (i.e., 18:2/16:1 and 18:3/16:0) could not be resolved via MS imaging alone, it was unclear whether these isomers differed in their abundance or cellular localization. Therefore, to address this question, MS/MS imaging experiments were performed in 5 μm high-spatial resolution to distinguish the structural isomers of PG 34:3, and determine whether their relative quantification or distribution was affected by genotype and/or leaf development. Figure 5 compares the relative abundance of PG 18:3/16:0 and PG 18:2/16:1 species that were extracted from MS/MS imaging datasets. For each genotype, PG 18:2/16:1 occurs at minimal levels in the basal zone (~5%), and the abundance increases along the developmental gradient and comprises 30-40% of PG 34:3 at the distal end. Mirroring the above change in PG 18:2/16:1, PG 18:3/16:0 accumulation decreases from ~95% to 60% across the developmental gradient of the leaf. The genotypic differences among these PG molecular species are minor, PG 18:2/16:1 reaches slightly higher levels in the distal section of B73 compared to Mo17xB73 or Mo17 ($p = 0.02$ and 0.03, respectively).

In Figure 6, five micron high-spatial resolution MS/MS images acquired at the midpoint zone show the localization patterns of the two individual isomers that constitute PG 34:3. Irrespective of the genotypes imaged, the cellular localization of PG 18:2/16:1 is evenly distributed between M and BS cells. In contrast, the cellular localization of PG 18:3/16:0 is mostly concentrated within BS cells. This divergence between distributions of PG 18:2/16:1 and PG 18:3/16:0 is in contrast to the localization of PG 32:0 (16:0/16:0) and PG 32:1 (16:0/16:1). As seen in Figure 4, PG 16:0/16:1 is mostly present in M cells, whereas PG 16:0/16:0 distribution differs among genotypes with localization to mostly BS cells in B73 and B73xMo17, and both M and BS cells in Mo17 and Mo17xB73. It suggests that the occurrence of the 16 carbon fatty acid in these lipids is not the only factor that determines their localization.

Table 1 summarized the cell-specific localization of PG molecular species for the four genotypes from Figures 4 and 6 (detailed data in Table S1). Quantitative analysis was performed for the intensity ratio of these PG molecular species in unit areas of BS vs M cells as shown in the parentheses in Table 1, which confirms the visual localization of these species. Combined altogether, there is a common trend in that FA16:0-containing PGs (i.e., PG 16:0/16:0, PG 18:3/16:0, PG 18:2/16:0) accumulate preferentially in BS cells, and FA16:1-containing PGs (i.e., PG 16:0/16:1, PG 18:3/16:1) are preferentially concentrated in M cells, although PG 18:2/16:1 is present almost evenly between M and BS cells. These contrasts occur regardless of genotype, except for PG 16:0/16:0, which uniquely shows genotypic differences, evenly distributed between M and BS cells in Mo17 and Mo17xB73 but more concentrated in BS cells in B73 and B73xMo17.

DISCUSSION

Physical separation of different cell types can be performed for lipidomic, transcriptomic, or proteomic analysis, but the sample processing involved can result in undesirable degradation or cross-contamination of the materials. MALDI-MSI provides distinct advantages for the visualization of metabolite distributions within intact tissues and cellular environments. This is especially important because the molecular distributions of metabolites or lipids may not always be the same, even among the same cell types. For example, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc) and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (HMBOA-Glc) are known to be present in M cells of maize, but MALDI-MSI revealed that they are present only in M cells between each pair of vascular bundles (Korte, et al. 2015). This is also observed in the current dataset for all four genotypes (Figure S10, S11).

The mechanisms underlying tissue development and function are fundamental questions in cellular biology. Maize is an excellent model to study the establishment of spatially distributed metabolic functions in which metabolism is facilitated by specialized cellular differentiation and cellular arrangement. In maize leaves, this is specifically associated with the differentiation of two types of photosynthetic cells, the bundle sheath and mesophyll, that are characteristic of the Kranz anatomy (Laetsch 1974), facilitating C4 photosynthetic photoautotrophy (Edwards *et al.* 2001, Hatch 1987, von Caemmerer and Furbank 2003). The chloroplasts within BS and M cells harbor different metabolic capabilities and express a different ultrastructure: the BS chloroplasts contain Rubisco and catalyze the Calvin cycle for CO_2-fixation, whereas these capabilities are absent from the chloroplasts of M cells (Nelson and Langdale 1992). In contrast, PSII and granal stacks are absent from BS chloroplasts (Woo *et al.* 1970).

Taking advantage of the MSI capabilities that we have developed over the past eight years, here we directly tested the hypothesis that the lipidomes of M and BS cells in maize are

differentially affected by cellular development, and that this developmental program is genetically controlled, as determined by the state of the maize genome. Specifically, we applied high spatial-resolution MSI (performed at either 5- or 10-micron resolution) on four developmental stages from leaves of four different maize genotypes, and compared the cellular location and relative abundances of two major anionic thylakoid lipids, SQDG and PG, within mesophyll and bundle sheath cells.

Typical of monocot leaves, the cellular differentiation that establishes the Kranz anatomy is linearly arranged from the base to the tip of the maize leaf, greatly facilitating its molecular and cellular study (Nelson and Langdale 1989). Recent studies have revealed the dynamic nature of cellular differentiation as changes in the transcriptome and proteome along the developmental gradient of the leaf (Li, et al. 2010). These analyses have uncovered a programmed change in the pattern of gene expression along the developmental length of the leaf, that parallels known morphometric alterations in cellular differentiation that marks the establishment of the Kranz anatomy (Li, et al. 2010). Specifically, in the basal section of the leaf, which is associated with the zone of cell division and elongation, expressed genes are markedly enriched for functions encoding for protein, DNA and cell wall biosynthesis. In the proximal section of the leaf that is associated with the cellular transition from sink to source tissue, the expressed genes are associated with photosynthetic machinery and secondary wall biosynthesis functions. Subsequently in the distal portion of the leaf, which is fully photoautotrophic, expressed genes associated with photosynthetic reactions, including the Calvin cycle, are enriched.

Similar transcriptomic (Tausta, et al. 2014) and proteomic (Majeran, et al. 2008) studies have specifically profiled the gene expression programs of BS and M cells along the developmental gradient of the maize leaf. These studies demonstrated the dynamic differences in

gene expression between the two photosynthetic cell types, with more than twice the number of differentially expressed genes at the midpoint section of the leaf as compared to the mature leaf tip. These molecular profiling studies identified differential expression patterns between M and BS cells, including those of photosystems I and II proteins, and thylakoid and envelope membrane proteins that function as metabolite transporters (Manandhar-Shrestha, et al. 2013). These molecular differences between M and BS cells are consistent with morphological and functional differences between these two cell types, particularly associated with the two chloroplast populations that differentiate the two cell types. These attributes particularly distinguish the photosynthetic capabilities and thylakoid membrane ultrastructures of the two chloroplast types (Hatch 1987).

Integrating the recent data on the dynamics of the transcriptomic and proteomic profiles with the ultrastructural changes associated with the developmental gradients of the two photosynthetic cell types, led to the hypothesis that there will be lipidomic differences between these two cell types and these will differentially affect the developmental gradient of the leaf. Indeed, earlier physical fractionation-based lipid analyses of isolated chloroplasts provided indications of such lipidome differences among chloroplasts of M and BS cells (Edwards and Black 1971, Nishihara, et al. 1980, Poincelot 1973, Woo, et al. 1970). However, conclusions from these earlier studies were confounded by the use of fractionation methods that included slow digestion processes, and were susceptible to cross-contamination of the M and BS cell fractions (Edwards and Black 1971, Kanai and Edwards 1973, Majeran, et al. 2005, Nelson and Langdale 1992, Poincelot 1973)**.** They were further confounded by the use of more complex analytical technologies, which often needed enzymatic digestion with undefined reagents (e.g., digestion of cell wall components with crude fungal derived hydrolytic enzyme preparation) or

chemical modification reactions of the tissue or the isolated lipids (e.g., use of positional-sensitive lipases or chemical reagents to characterize lipid molecular species) (Christie and Han 2012, Kuksis *et al.* 1983, Siebertz *et al.* 1979). Application of modern advances in mass spectrometry has more recently provided enormous progress in the understanding of acyl lipid profiles and lipid metabolism in plants (Gasulla *et al.* 2013, Li-Beisson *et al.* 2013, Ramadan *et al.* 2014, Riedelsheimer *et al.* 2013, Tarazona *et al.* 2015, Vu *et al.* 2014, Welti *et al.* 2002).

SQDG and PG are chloroplast lipids that are crucial for maintaining chloroplast structure and function, including the stability of the PS II complex in the thylakoid membranes (Kansy *et al.* 2014, Yu and Benning 2003), and they are essential for growth and development of photoautotrophs (Yu and Benning 2003). PG could be distinguished from SQDG by the fact that it has at least one 16-carbon fatty acyl chain, either 16:0 or 16:1. We could not determine whether these 16-carbon acyl-chains occur at the sn-1 or sn-2 position, but earlier studies indicate that both 16:0 and 16:1 prefer the sn-2 position, with 16:1 having the priority if both acyl chains are present (i.e., PG 16:0/16:1) (Nishihara, et al. 1980). One clear difference between our data and the earlier study is the presence of PG 18:2/18:3 localized to BS cells (Nishihara, et al. 1980). According to the extract analysis, we do observe PG 36 species but in much low abundance (Figure S15). Hence, the difference is most likely associated with the difference in genotype (L. var. Honey Buntum) and/or age of tissue (45 day old plants).

The relative abundances of SQDG molecular species determined by MSI is consistent with earlier bulk-extract analysis of this class of lipid; namely, 16:0- and 18:3-containing SQDG lipids account for the majority of this lipid class (Kenrick and Bishop 1986) with 80-90% being SQDG 32:0 and SQDG 34:3. There are only minor differences in the abundance of these lipids among the four genotypes evaluated; however, development dramatically affects their

abundance. Particularly, the most abundant SQDG 34:2 in the basal zone is replaced by SQDG 34:3 as leaf develops. In the 3rd leaf that we assessed for molecular imaging, this cellular differentiation occurs as the leaf expands through the ligule of the 2nd leaf, and becomes directly exposed to illumination. This is marked by the differentiation of proplastids to different types of chloroplasts that are non-uniformly distributed between BS and M cells, and this differentiation is associated with an increase in the size and number of chloroplasts per cell. At this differentiation of the M and BS chloroplasts, granal stacks begin to develop in M chloroplasts, but not in BS chloroplasts (Majeran *et al.* 2010). Therefore, the observed changes in the distribution of the different molecular species of SQDG may suggest that SQDG 34:3 is mostly associated with thylakoid membranes, whereas SQDG 34:2 is a major component of the chloroplast envelopes.

In the case of PG, PG 34:2 is the dominant molecular species in the basal zone of the leaf, accounting for 80-90% of this lipid class, with essentially no difference among the inbreds and hybrids evaluated (Figure 3). As leaf development progresses, the abundance of other PG molecular species (PG 32:0 or PG 34:3) increase and they become most dominant at the midpoint and distal end sections. This is consistent with earlier work that showed the increase of 18:3 and the decrease of 18:2 fatty acyl composition of PG across the developmental gradient of the maize leaf (Roughan 1985). Using the same logic as discussed above for SQDG molecular species, these findings may suggest that PG 34:2 is mostly associated with chloroplast envelopes, and PG 32:0 and PG 34:3 molecular species are primarily associated with thylakoid membranes. The relative abundance of PG molecular species, however, continuously changes, at least up to the midpoint (Figure 3B), whereas SQDG shows only minimal change after the proximal zone (Figure 3A). Furthermore, 16:1 fatty acyl composition of PG continuously increases as the leaf

matures, manifested by the increase of PG 32:1 (Figure 3B) and PG 34:3 (18:2/16:1) (Figure 5) from the basal to distal zones of the leaf. This suggests the importance of PG molecular species, specifically with 16 carbon fatty acyl chains, for the functional development of chloroplasts, not merely anatomical differentiation mostly dictated by 18:2 and 18:3. In addition to the developmental effect on the relative abundances among PG molecular species, there is also a genotype dependence on the distribution of these lipids. This difference among the genotypes is especially prominent at the proximal end of the leaf, and becomes indistinguishable as the leaf expands into the mature stages.

Of significance, there is a difference in the localization of PG molecular species between BS and M cells, which appears to be dependent on the fatty acyl chains, and it is also a trait that is partially influenced by genotype. Such a heterogeneity was previously suggested by the lipid analysis of physically separated BS and M cells (Nishihara, et al. 1980). This differential localization is not exclusive but rather preferential, and major PGs are present in both BS and M cells. As summarized in Table 1, PGs with FA16:0 and FA16:1 prefer to localize in BS and M cells, respectively, for all genotypes. The preferential localization of FA16:1-containing PGs in M cells and their increased accumulation in later stages of leaf development may indicate their importance to the development of the PSII complex and granal stacks, which are localized in M cells and most enriched in the distal region of the leaf (Li, et al. 2010, Majeran, et al. 2010). Some PGs are much more homogeneously localized between the two cell types, specifically PG 18:2/16:1 in all four genotypes (Figure 6) and PG 16:0/16:0 in Mo17 and Mo17xB73 (Figure 4). Homogeneous distribution of these PG molecular species may suggest the importance of PG 18:2/16:1 and PG 32:x in both BS and M cells. Considering the importance of 16:1 containing PGs, it is reasonable to hypothesize that a corresponding desaturase would be differentially

expressed in large scale proteomics or transcriptomics datasets across, especially as leaf development and/or between different cell types (Li, et al. 2010, Majeran, et al. 2008). In Arabidopsis, fatty acid desaturase 4 (FAD4) is known to be involved in the formation of $\Delta^{3\text{-trans}}$ FA16:1 at the sn-2 position of PGs (Gao *et al.* 2009). The maize genome includes two homologs of FAD4 in NCBI BLASTP search, i.e., GRMZM2G175401 and GRMZM2G097509, and both of them are expressed in shoots, especially at the tip of stage 2 leaf of V5 and V7, respectively (Sekhon *et al.* 2011). Furthermore, GRMZM2G175401 is shown to have a higher expression in M cells than BS cells according to supplementary data of Li et al. (Li, et al. 2010). However, the correlation between the content of 16:1t in PGs and FAD4 is obscure as the reduction of 16:1t has been also reported for other mutants in PG biosynthesis such as *pgpp1-1* and *pgp 1* (Babiychuk *et al.* 2003, Kobayashi *et al.* 2015, Lin *et al.* 2016).

In summary, the work described herein demonstrates that high-resolution MALDI-MSI analysis can be directly applied to multicellular plant tissues to uncover cell-specific metabolic biology that has not been possible using traditional metabolomics methodology. This capability of *in situ* imaging is specifically valuable for metabolites and lipids because of their highly dynamic nature compared to transcriptome or proteome. Combined with other single cell "-omics" technologies (Dai and Chen 2012, Shalek *et al.* 2013, Wang and Bodovitz 2010), high-resolution MSI will allow for enhanced systems biological understanding in single cell-level in the foreseeable future.

EXPERIMENTAL PROCEDURES

Plant growth and sample preparation

The overall experimental workflow is illustrated in Figure S1. Maize kernels (*Zea mays* L. inbreds B73 and Mo17, and reciprocal hybrids B73xMo17 and Mo17xB73) were planted in soil, and grown in a climate-controlled greenhouse at 30% humidity under a diurnal cycle of 16 h light and 8 h dark at 27°C and 24°C, respectively. Seedlings were harvested at approximately 1pm local time, 11-13 days after planting, when the length of the third leaf was 14-18 cm, as measured from the second ligule. Sections from four positions along the developmental gradient of leaf 3 (Figure S2) were collected from three individual plants of each genotype, at similar positions as selected by Li *et al* (Li, et al. 2010). The etiolated basal zone leaf section was collected as a cross-section through the sheathed basal zone of leaf 3 within the whorl, 1.5 cm below leaf ligule 2. The second section of leaf 3, labeled as the proximal section, was collected 1.5 cm above the position of the ligule 2 and at the point of emergence from the whorl, where the leaf begins to unfold and becomes fully exposed to direct illumination. The third section of leaf 3, labeled as the midpoint, was collected halfway between the proximal section and the leaf tip. The fourth section of leaf 3, labeled as the distal section, was collected ~2 cm from the tip of the leaf and approximately 12-16 cm from the base.

Tissue samples were cryo-sectioned and prepared for MALDI-MSI as described previously (Korte and Lee 2014). Briefly, fresh maize leaf sections were placed in a cryo-mold, then submerged in gelatin (10% w/v solution) and immediately frozen with liquid nitrogen. The molds were transferred to a cryostat (CM1850, Leica Microsystems; Buffalo Grove, IL, USA) that was pre-chilled to -20°C, and the samples were allowed to thermally equilibrate for 30 minutes. Leaf tissue was cryo-sectioned at 10 μm thickness and was then collected with Cryo-Jane tape (Leica Biosystems), and attached to pre-chilled glass slides. The prepared slides were placed onto a chilled aluminum block and were vacuum dried while gradually warming to room

temperature. After acquiring optical microscope images (Axio Zoom.V16, Carl Zeiss; Thornwood, NY), the dried tissues were subjected to matrix deposition by sublimating 1,5-diaminonaphthalene (97%, Sigma-Aldrich, St. Louis, MO, USA) (Hankin *et al.* 2007) at 140°C for 4 minutes at a pressure of ~50 mtorr.

For fluorescence image shown in Figure S12, a leaf tissue was cryo-sectioned at 10 μm thickness and collected on a charged glass slide (Fisher Scientific; Waltham, MA, USA) by thaw mounting. Fluorescence image was obtained with an Olympus BX-60 microscope with Jenoptik C-5 camera. A consecutive cross-section was collected with Cryo-Jane tape to compare the MS image of chlorophyll *a*.

Mass spectrometry analysis

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 355 nm frequency tripled Nd: YAG laser (UVFQ, Elforlight Ltd.; Daventry, UK). The laser energy used was 83-84% (~1 μJ/pulse) at a 60 Hz repetition rate. Laser optics is similar to previously described (Korte, et al. 2015), but the focus lens was further reduced to 60 or 75 mm and a commercial 10X or 5X beam expander (Thorlabs, Newton, NJ, USA) was used to reduce the laser spot size. The laser spot size was 9-11 μm for 10 μm-resolution imaging with a 5X beam expander and 6-7 μm for 5 μm-resolution imaging with a 10X beam expander; thus, 5 μm-resolution imaging was acquired using an oversampling method. TunePlus and Xcalibur software (Thermo Scientific) were used to define imaging parameters and to acquire data, respectively. Mass spectra were acquired with 10 laser shots per spectrum in

negative mode using an Orbitrap mass analyzer (resolution of 30,000 at *m/z* 400) for *m/z* scan range of 100-1000.

All lipids were detected as deprotonated, $[M-H]^-$. MS images were generated using ImageQuest (Thermo) with a mass window of ±0.003 Da and with normalization to the total ion count (TIC) for Figures 1, 2, S4-S7, and without normalization for the rest of the figures. Overlaying of MS images with the optical microscope images were made using the software module of MATLAB, Image Processing Toolbox 9.3 (MATLAB 2015). MSiReader v.0.09 (Robichaud *et al.* 2013) was used to obtain the ion signal ratios of PG molecular species between BS and M cells normalized to each cell area. First, the optical images and MS images were overlaid using the MSiImage tool. Then, the region of interest (ROI) for the bundle sheath and mesophyll cells were selected using the polygon drawing tool and the spectra intensity data for each ROI was exported for all PG species. The total intensity of each PG species at each ROI was then normalized to the area of the ROI for each cell type.

MS/MS imaging was performed using the ion trap analyzer for selected ions using the same conditions as described for MS imaging. An isolation width of 2.0 Da and normalized collision energy of 35 were used. MS/MS images were generated using MSiReader with a mass window of ±0.2 Da and no normalization to the TIC. Quantitative comparison was made by averaging metabolite intensities over the entire imaging area of the maize leaf that contained both mesophyll and bundle sheath cells. Three or more biological replicates of each genotype were analyzed, and the average is reported with standard deviation. Student T-tests were performed to calculate pairwise p-values.

Lipid Extraction and Direct-Infusion ESI-MS

Total lipids were extracted from maize leaf (inbred B73) as described in Shiva et al. (Shiva *et al.* 2013). Lipid samples were then purified by two-phase partitioning with chloroform and 1 M KCl, dried under N_2 and resuspended in chloroform, and stored in -20°C until MS analysis. Three biological replicates were analyzed, and the average is reported with standard deviation. Total lipid extracts were analyzed by direct-infusion ESI-MS on a Fourier Transform Ion Cyclotron Resonance mass spectrometer (7T SolariX FT-ICR MS; Bruker Daltonics, Bremen, Germany).

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Short Supporting Information Legends

Figure S1. Overall workflow for MALDI-MSI of maize leaves

Figure S2. Illustration of four positions along the developmental gradient of maize leaf 3.

Figure S3. Optical microscope images of transverse sections of a B73 maize leaf.

Figure S4. Ten-micron resolution MS images of B73 inbred at four developmental stages.

Figure S5 Ten-micron resolution MS images of B73xMo17 hybrid at four developmental stages.

Figure S6. Ten-micron resolution MS images of Mo17xB73 hybrid at four developmental stages.

Figure S7. Ten-micron resolution MS images of Mo17 inbred at four developmental stages.

Figure S8. MS/MS spectra of SQDG 32:0, SQDG 34:3, and SQDG 36:6.

Figure S9. MS/MS spectra of PG 32:0, PG 34:2, and PG 34:3.

Figure S10. Five-micron resolution MS images at the midpoint cross-section of leaf 3.

Figure S11. Five-micron resolution MS images at the distal cross-section of leaf 3.

Figure S12. Comparison of chlorophyll *a* between MS and fluorescence images.

Figure S13. MALDI-MS spectra of soy PG standard with chlorophyll *a* only and chlorophyll *a* and DAN as matrix.

Figure S14. MALDI MSI analysis of five cross-sections of maize leaf.

Figure S15. Comparison of PG and SQDG species from total lipid extract obtained by ESI-FTICR MS and MALDI-MS.

Table S1. Quantitative analysis for cell-specific localization of PG species.

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Figure Legends

Figure 1. Ten-micron resolution MS images of representative lipids at four developmental sections of the 3rd seedling leaf from inbreds B73 (Top panel) and Mo17 (Bottom panel). The MS images are of PG 32:0 (*m/z* 721.503; E-H, E'-H'), PG 32:1 (*m/z* 719.480; I-L, I'-L'), SQDG 34:3 (*m/z* 815.501; M-P, M'-P'), and chlorophyll *a* [sum of fragments at *m/z* 591.261 (pheophorbide *a*), 613.232 (chlorophyllide *a*), and 870.566 (pheophytin *a*); Q-T, Q'-T']. The ion signals are normalized to the total ion count and the maximum value of $1.3x10^{-2}$ was used for all the images. Scale bars represent 50 μm.

Figure 2. Ten-micron resolution MS images of representative lipids at four developmental sections of the 3rd seedling leaf from reciprocal hybrids B73xMo17 (Top panel) and Mo17xB73 (Bottom panel). The ion signals are normalized to the total ion count and the maximum values used were $3.0x10^{-3}$, $3.0x10^{-3}$, $1.3x10^{-2}$, and $1.3x10^{-2}$, for PG 32:0, PG 32:1, SQDG 34:3, and Chl *a*, respectively. Scale bars represent 50 μm.

Figure 3. Quantitative comparison of SQDG (Left) and PG (Right) molecular species from MS images of the distal (A, A'), midpoint (B, B'), proximal (C, C'), and basal zone (D, D') sections of maize leaves in four genotypes.

Figure 4. Five-micron resolution MS images of each PG molecular species at the midpoint and distal sections of leaf 3 from the four genotypes overlaid with optical images. Scale bars represent 50 μm; note scale bar is bigger for Mo17 and Mo17xB73.

Figure 5. Quantitative comparison of the distribution of PG 18:3/16:0 and PG 18:2/16:1 as affected by leaf development among the four genotypes. These data were extracted from MS/MS imaging data sets of PG 34:3.

Figure 6. Five-micron resolution MS/MS images of two structural isomers of PG 34:3 at the midpoint section of maize leaves. The ion abundances for PG 18:3/16:0 is determined from the sum of $[M-16:0]^-$, $[M-(16:0 - H_2O)]^-$, and $[M-(16:0+glycerol)]^-$ and PG 18:2/16:1 is determined from $[M-16:1]^-$, $[M-(16:1-H_2O)]^-$, and $[M-(16:1+glycerol)]^-$. The scale bar corresponds to 50 μm.

Table 1. Summary of cell-specific localization of PG species.

	B73	B73xMo17	Mo17xB73	Mo17
PG 32:1 (**16:0/16:1**)	**M** (0.66-0.72)	**M** (0.23-0.70)	**M** (0.01-0.93)	**M** (0.09-0.78)
PG 32:0 (**16:0/16:0**)	**BS** (2.8-9.3)	**BS** (1.9-2.8)	**M and BS** (0.70-1.4)	**M and BS** (0.71-1.5)
PG 34:4 (18:3/**16:1**)	**M** (0.46-0.91)	**M** (0.14-0.48)	**M** (0.15-0.44)	**M** (0.22-0.98)
PG 34:3 (18:2/**16:1**)	**M and BS** (1.1-2.4)	**M and BS** (1.2-2.8)	**M and BS** (0.93-1.3)	**M and BS** (1.1-1.4)
(18:3/**16:0**)	**BS** (2.2-3.4)	**BS** (2.3-4.8)	**BS** (1.6-3.2)	**BS** (2.2-5.4)
PG 34:2 (18:2/**16:0**)	**BS** (5.0-83)	**BS** (6.4-97)	**BS** (2.9-87)	**BS** (6.9-57)

* The numbers in parenthesis represent the range of ion signal ratios in BS vs M cells per unit pixel area obtained from three replicates of midpoint sections. The detailed analysis is shown in Table S1.