2-2016

Matrix assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) for direct visualization of plant metabolites in situ

Drew Sturtevant
University of North Texas

Young-Jin Lee
Iowa State University, yjlee@iastate.edu

Kent D. Chapman
University of North Texas

Matrix assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) for direct visualization of plant metabolites in situ

Abstract
Direct visualization of plant tissues by matrix assisted laser desorption ionization-mass spectrometry imaging (MALDI-MSI) has revealed key insights into the localization of metabolites *in situ*. Recent efforts have determined the spatial distribution of primary and secondary metabolites in plant tissues and cells. Strategies have been applied in many areas of metabolism including isotope flux analyses, plant interactions, and transcriptional regulation of metabolite accumulation. Technological advances have pushed achievable spatial resolution to subcellular levels and increased instrument sensitivity by several orders of magnitude. It is anticipated that MALDI-MSI and other MSI approaches will bring a new level of understanding to metabolomics as scientists will be encouraged to consider spatial heterogeneity of metabolites in descriptions of metabolic pathway regulation

Keywords
Food biotechnology, Plant biotechnology

Disciplines
Biotechnology | Chemistry | Plant Biology

Comments
NOTICE: This is the author's version of a work that was accepted for publication in Current Opinion in Biotechnology. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Current Opinion in Biotechnology, 37, February 2016, doi: 10.1016/j.copbio.2015.10.004.

Title: Matrix Assisted Laser Desorption/Ionization-Mass Spectrometry Imaging (MALDI-MSI) for Direct Visualization of Plant Metabolites *In Situ*

Authors and Affiliations: Drew Sturtevant[1], Young-Jin Lee[2,3], and Kent D. Chapman[1]

Center for Plant Lipid Research and Department of Biological Sciences, University of North Texas, Denton, Texas 76203, United States[1]

Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States[2]

Ames Laboratory, US Department of Energy, Ames, Iowa 50011, United States[3]

Postal Addresses:

University of North Texas, Department of Biological Sciences, 1155 Union Circle #305220, Denton, Texas 76203, United States[1]

Iowa State University, Department of Chemistry, 35A Roy J. Carver Co-Laboratory, Ames, Iowa 50011, United States[2]

E-mail Addreses:

Drew Sturtevant; drewsturtevant@my.unt.edu

Young-Jin Lee; yjlee@iastate.edu

Kent D. Chapman; chapman@unt.edu

Corresponding Author: Kent D. Chapman

Highlights:

Plant chemical organization is visualized directly using MALDI-MSI.

MALDI-MS imaging of plant tissues reveals unforeseen analyte compartmentalization.

Analyte heterogeneity has been validated by other MS and biochemical analyses.

Novel instrument modifications have driven spatial and chemical resolution to unprecedented levels.

Abstract

Direct visualization of plant tissues by matrix assisted laser desorption ionization- mass spectrometry imaging (MALDI-MSI) has revealed key insights into the localization of metabolites *in situ.* Recent efforts have determined the spatial distribution of primary and secondary metabolites in plant tissues and cells. Strategies have been applied in many areas of metabolism including isotope flux analyses, plant interactions, and transcriptional regulation of metabolite accumulation. Technological advances have pushed achievable spatial resolution to subcellular levels and increased instrument sensitivity by several orders of magnitude. It is anticipated that MALDI-MSI and other MSI approaches will bring a new level of understanding to metabolomics as scientists will be encouraged to consider spatial heterogeneity of metabolites in descriptions of metabolic pathway regulation.

Introduction

Plant based agricultural products and chemical byproducts contribute hundreds of billions dollars annually to the global economy (www.faostat.fao.org), and originate from a diversity of grain, oilseed, fiber, and numerous other crops worldwide. Optimal production of chemicals derived from these crops requires an underlying understanding of the synthesis and metabolism of these compounds, especially in support of widespread metabolic engineering efforts to generate sustainable, bio-based products. An improved understanding of plant metabolism relies on analytical capabilities for the accurate identification and quantification of metabolites, which metabolomics has largely addressed through the development of sophisticated separation techniques, mass spectrometry approaches, and computational tools [1]. Analysis of chemical/tissue extracts by these methodologies provides excellent qualitative and quantitative information about chemical composition, but provides little or no data regarding the original spatial distribution of metabolites *in situ*. A number of years ago, the Caprioli group pioneered the development of mass spectrometry imaging for localizing molecules of interest directly on the sections of mammalian tissues [2, 3]. This methodology, termed matrix assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI, also referred to as imaging mass spectrometry (IMS)), has since been expanded to visualize molecules directly in plant tissues and surfaces for the localization of lipids [4, 5, 6, 7], proteins [8], secondary metabolites [9, 10, 11, 12, 13], and various small molecules [14, 15, 16, 17] at unprecedented spatial and chemical resolution.

The last several years have seen increasing applications of MALDI-MSI to plant tissues which have been enabled, in part, by novel instruments and matrices to improve spatial resolution and chemical coverage [18, 19], streamlined matrix and sample preparation [20], and easily accessible open-source image processing free-ware [21, 22, 23]. These and other advances have enabled scientists to not only generate high resolution chemical images of plant tissues, but also to combine spatial metabolite data with further quantitative MS studies and gene

expression analysis. In addition to MALDI-MSI there are other MSI platforms being utilized for MS imaging of plant tissues *in situ*, including desorption electrospray ionization (DESI-MS), laser ablation electrospray ionization (LAESI-MS) and secondary ion mass spectrometry (SIMS), and

several salient reviews of these platforms in the context of plant MSI are available [6, 24, 25, 26]. Here we highlight the most recent applications of MALDI-MS imaging in plant tissues (Table 1) for metabolite localization *in situ*. Efforts examined here have also begun to address next-level biological questions in biochemical and metabolic contexts. It is anticipated that this new dimension of spatial analysis will provide important insights into the function, regulation and manipulation of plant metabolism.

MALDI-MSI: a Procedural Overview

A typical experimental workflow for MALDI-MSI is provided in Fig. 1. First, plant tissues are flash-frozen (with or without fixation) in an embedding media, often gelatin, and then cryo-sectioned and lyophilized for tissue imaging; or depending on the location of the analytes of interest and the nature of the tissue, intact plant parts can be lyophilized and imaged directly for surface metabolites. A chemical matrix, to promote desorption and ionization, is applied by either a sprayer/nebulizer or by solvent-free sublimation [27, 28]. After matrix deposition, the sample plate is inserted into the instrument and the user defines experimental parameters including laser energy, plate movement step size, and a selected area of the tissue to be scanned for imaging. At every x and y coordinate within the scanning area, energy provided by the laser generates a plume of ions, which are then guided to the mass spectrometer and produce a mass spectrum. The resulting spectra at each location are used to reconstruct MS images for ions of interest by converting the ion's intensity at every coordinate into a color scheme.

Each step of sample preparation is critical in MSI to maintain original molecular distributions and obtain best sensitivity. Selection of matrix and its deposition method are additional considerations, which must be taken into account as they can dramatically influence spatial resolution and the classes of molecules to be analyzed. For example, while sublimation of matrix provides very small matrix crystal size (<~1 μm) needed to generate high resolution MS

images, solvent based matrix application methods, which generally produce markedly larger crystal sizes (10-100µm), have been demonstrated to increase the number of metabolites detected [28, 20].

Localization of Molecules in Plant Tissue Sections and on Tissue Surfaces

Lipids

The biochemical characteristics of lipids lend themselves to analysis by MALDI-MSI and not surprisingly are one of the most common types of molecules to be spatially mapped using MSI. Phospholipids, comprising the lipid-bilayer of cell membranes, and triacylglycerols (TAGs), ~30% mass of oil seeds, have been visualized by MSI. One of the first comprehensive MALDI-MSI lipidomics studies in plant sections examined the spatial distributions and composition of the major and minor storage and membrane lipids (e.g., TAGs, phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), and phosphatidic acid (PA) species) in embryos of upland cotton, *Gossypium hirsutum* [4]. Mass spectrometry images of longitudinal- and cross- sections of these embryos, revealed that polyunsaturated, monounsaturated, saturated, and cyclic lipid species of each lipid class were differentially enriched in the cotyledons and embryonic axis of the embryo. These differences in tissue compartmentalization of phospholipid and TAG molecular species suggested that metabolic pathways for these metabolites occurred in a heterogeneous distribution within embryo tissues, pointing to a biochemical complexity that had been previously unappreciated. Similar studies with seeds of genetically-engineered lipid mutants of *Camelina sativa* [5], siliques of *Arabidopsis thaliana* [6], genetically modified, high-oleic containing cottonseeds [7], and *Persea americana* (avocado) mesocarp [29] have all revealed marked tissue-level heterogeneity in phospholipid and storage lipids indicating that the ***location*** of metabolites is an important consideration when delineating plant metabolism. Importantly, the overall mol% abundances of lipid species within lipid classes revealed by MS images in tissue sections agreed well with quantification of these same lipids by conventional analyses of total lipids extracts. Indicating that the MALDI-MSI provides an authentic representation of the relative proportions of lipid molecular species within a lipid class, and also provides additional spatial information about where these lipids are localized in tissues. In

attempts to produce new triacylglycerol compositions in *Camelina sativa* seeds, it was clear that compartmentalization of metabolism can represent potential bottle necks to achieving

desired outcomes for accumulation of highest percentages of metabolites [5], and so analyses of spatial distribution can reveal important information to inform metabolic engineering strategies.

Secondary Metabolites

Plant secondary metabolites represent a broad class of structurally and functionally diverse compounds which play vital roles in plant defense against herbivores and microbial/fungal infection, act as signaling molecules, and are utilized for pollinator attraction. In recent years these compounds have received increased attention from the biomedical community for their potential as anti-cancer treatments as well as other benefits for human health. Several secondary metabolites have already been investigated using MALDI-MSI including: hortatines in mature barley seeds [10], flavonols and dihydrochalcones in Golden Delicious apple fruit sections [30], and aporphine alkaloids in the roots of Himalayan Mayapple [9]. Lignan and cyanogenic glucoside-related metabolites, coveted for their antioxidant activity, were investigated by MALDI-MSI in developing *Linum usitatissimum* (flax) capsules and seed tissues. In the latter "multi-omics" study, the upregulation and localization of transcripts of genes involved in secoisolariciresinol derived glucoside (SDG) synthesis were correlated with the distribution of metabolites of SDG and 6a-hydroxymethylglutaric (6a-HMG)-SDG in MS images. Furthermore, molecular identities of these and other metabolites in this pathway were confirmed using ion mobility and MS/MS analyses [11]. Hence, MALDI-MSI reveals detailed, high-resolution chemical and spatial information about plant metabolites of a variety of types, and should emerge as an excellent tool in the detailed characterization of plant metabolism.

In an intriguing insect herbivore-plant interaction study, the metabolism of ingested glucosinolate, sinalbin, from *Sinapsis alba* (white mustard) leaves by *Athalia rosae* (turnip sawfly) was monitored using MALDI-MSI. MS images of longitudinal cryo-sections of these

larvae revealed the rapid sequestration and concentration of sinalbin in the haemolymph, rather than gut, as a strategy to detoxify ingested leaf material [13], a conclusion likely to be obtained only through MS localization and not through conventional analysis of tissue extracts.

Small-Molecule Metabolites

Characterization of spatial distribution of small molecules, molecular weight < 500 Da, by MALDI-MSI has remained a challenge due to ion suppression by intense matrix peaks and susceptibility to in-source fragmentation. The wide variety in chemical functionality is another significant challenge for these small molecules because of matrix-dependent ionization selectivity. Several plant metabolites of high importance fall into this range, notably the major plant hormones (e.g., abscisic acid, indole acetic acid, jasmonate, gibberellic acid, etc.), and have yet to be well described, or even analyzed at all using MALDI-MSI. Despite these challenges, recent efforts have been successful in MALDI-MSI analysis examining small molecules in plant tissue sections (Table 1), indicating the promise of expanding this technique to a wider range of small molecule metabolites.

Several new matrices with little or no interfering matrix peaks have been successfully applied in negative mode, such as 9-aminoacridine (9-AA) for glucosinolates in *Arabidopsis thaliana*, 1,8-bis(dimethyl-amino)naphthalene (DMAN) for organic acids, nucleic acids and small sugar molecules in *Medicago truncatula* root nodules, and 1,5-diaminonapthalene (DAN) for organic acids, amino acids, and benzoxazanoids in *Zea mays* leaf cross sections [12, 31, 17] . Among those, DMAN should be adopted with caution for MSI due to its vacuum instability. 9-AA has shown superiority for phosphate containing compounds whereas DAN has shown broad applicability for both small molecules and lipids [17]. Choice of matrix is often limited in positive mode because most positive matrices produce significant background peaks. Recently, nanoparticle based matrices have shown promise for positive mode application. In bacterial resistant rice leaves, metal-oxide nanoparticles TiO_2 and Fe_3O_4 were used to monitor metabolite accumulation of phytoalexins in response to infection by *Xanthomonas oryzae* bacterium, which was not possible with any other matrices [15].

Metabolic incorporation of stable isotope labels has a great potential to be used for the understanding of dynamics involved in metabolism; however, its utilization for MSI has been limited to date [32]. In an interesting recent study, MALDI-MSI and SIMS were used to

demonstrate the recycling of nitrogen by living plants from ^{15}N enriched dead plant materials into ^{15}N choline and phosphocholine [14]. Similar approaches could be applied for other metabolic flux imaging analyses such as uptake of herbicide, pesticides and nutrients, or perhaps even applied to metabolic labeling type studies.

On Plant Surfaces

One of the unique capabilities of MALDI-MSI is that ions can be desorbed/ionized directly off tissue surfaces. This approach has its foundation in earlier studies like those described by Jun et al., that used laser desorption ionization and colloidal silver to analyze the epicuticular lipids on the surface of *Arabidopsis* flowers, stems, and roots [33]. Surface analysis by MALDI-MSI had proven particularly advantageous for plant leaves, as the laser beam typically cannot penetrate into the thick cutin layers. Shroff et al. could selectively study glucosinolates on the surface of Arabidopsis leaves, distinguished from intracellular glucosinolates, and quantified using internal standards spotted on the tissue surface [12]. Detection of molecules directly off tissue surfaces by MALDI is certainly not limited to leaf tissues. Other groups have imaged glucosinolates on *A. thaliana* flowers and siliques [34], secondary metabolites in trichomes [35], defense induced synthesis of pinoresinol monogulcoside in pea pod endocarp [36], and the antibiome of roots with a developing biofilm of *Bacillus amyloliquefaciens* [37]. Due to the relative ease sample preparation, and the applicability of MSI to surface analysis by a variety of methods, imaging surfaces of plants should be a prolific area of research in years to come.

Technological Advances and Future Perspectives

Technological Advances

Several recent and notable technological advances have expanded the current capabilities of

modern MALDI MSI instrumentation. First, the modification of laser optics achieved the capability of < 10 μm laser spot sizes. A sampling size of this dimension can not only be used to map chemical heterogeneity by tissue type, but also has the potential to map heterogeneity on

cell-to-cell, or even organelle–to-organelle basis. The Spengler group achieved spatial resolution in the range of 3 μm using a close-up laser focusing in atmospheric pressure MALDI [38]. More recently, the Caprioli group has achieved the laser spot size down to 5 μm using modified laser optics in a commercial instrument [39] and 1 μm in transmission geometry [40]. The spatial resolution limits of MSI in plants, however, has been ~10 μm, mostly because of the difficulty to maintain high sensitivity in small sampling size. Nonetheless, the Lee group has recently demonstrated cellular/subcellular level resolution MSI for juvenile *Zea mays* leaf cross sections at 5 μm spatial resolution using a modified laser optics with 9 μm laser spot size coupled with oversampling [18]. Figure 2 shows the localization of flavonoids in the single cell layer of the upper and lower epidermis, and differential localization of phosphatidylglycerols (PG) between bundle sheath and mesophyll cells. In addition, subcellular level localization was demonstrated for certain compounds; i.e., sulfoquinovosyl diacylglycerol (SQDG) was localized to chloroplasts, and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside [DIMBOA-Glc], 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside [HMBOA-Glc] were reported to be localized to vacuoles. It was further of interest that DIMBOA-Glc and HMBOA-Glc were detected in vacuoles of mesophyll cells only between vascular bundles. These types of high-resolution, direct metabolite localization data simply are unable to be determined by other, conventional lipidomics analysis of leaf extracts no matter how comprehensive and thorough.

Another major advance was to improve sensitivity with secondary ionization [19]. Termed MALDI-2 this secondary step ionizes molecules commonly lost as neutrals during the first laser desorption/ionization event. For apple sections, MALDI-2 could detect dihexose (probably sucrose), localized in the hypanthium, and the phenolic glycoside, quercetin pentoside, localized in the skin, at almost two orders of magnitudes higher ion intensity compared to conventional MALDI. This MALDI-2 approach should not only increase the sensitivity and detection of low abundance metabolites, but should also enable further reductions in spatial resolution, since less energy accompanying smaller laser spot sizes will still yield higher

amounts of metabolites from the MALDI-2 event.

Finally, low throughput in typical high-resolution MSI experiments and lack of streamlined workflow for data analysis are two serious bottlenecks in MSI imaging; for example, it may take

as much as 10 hours for a few millimeter size tissue sections at 10 µm spatial resolution. Bruker recently commercialized a new MALDI-TOF MS with ten to fifty times faster than traditional mass spectrometers, 50 true pixels per second, using a 10 kHz laser, scanning laser mirrors, and synchronized plate movement. Additionally, after a lengthy MS experiment researchers often manually generate each MS image of interest to find correlations, which is considerably time consuming. Recent efforts in computational tools may accelerate this data processing. For example, commercial software is now available that can automatically perform statistical analysis correlating the *m/z* ions that have similar image patterns (www.scils.de). Taken together, advances in spatial resolution, sensitivity, and scanning speed will continue to support new discoveries about localizations of metabolites in tissues, cells and organelles in studies of plant metabolism.

Future Perspectives

Efforts in the last 3 years, as well as those on which recent studies are based, have made remarkable progress in advancing MALDI-MSI for visualizing plant metabolites *in situ*. Mechanical and physical instrument development is very close to the ultimate level, and upfront technology, e.g., development of new matrices and sample preparation, is expected to evolve and improve in the coming years. Quantification in MSI is still a major hurdle, which is not easy to overcome because of the complexity of this problem; e.g., local environment or tissue specific ion suppression, differential extraction efficiency, and lack of understanding of MALDI mechanisms. Some progress has been made in the last few years [41], and improvements are likely to continue in the near future. With all the progress and trends, it would not be surprising within the next decade to find this technology become a routine and essential tool that is commonly available to a wide range of plant biologists. Important contributions have been made to date for major macromolecules to minor secondary metabolites, describing previously unknown heterogeneity of these molecules and then validating *in situ* visualization with genetic and/or complementary MS analyses. While it is clear

that localization of metabolites in biological organisms is far more heterogeneous and complex than was once thought, it is really only through direct visualization of molecules within tissues

that this heterogeneity has been revealed. Outstanding questions in MALDI-MSI include, but are not restricted to, the following: What is the physiological, biochemical, or developmental significance of heterogeneity of tissue metabolites? How are differences in distribution established and can this be further addressed with complementary techniques such as *in-situ* hybridization of mRNA or MALDI-MSI of metabolic pathway enzymes? Can three-dimensional MALDI-MS images be developed to improve the overall perspective of metabolite heterogeneity? What are the technical advances that need to be made to visualize more small molecules *in situ* with confidence? What ways can MALDI-MSI be used to trace metabolism over time? Can quantification be routinely achieved with MALDI-MSI? Answers to these and other questions will help move MALDI-MSI, and other approaches to localize metabolites directly in plant cells and tissues, to the forefront in biochemical research, and will continue to promise a visual perspective of metabolism with profound and far reaching implications.

Acknowledgements: Support for the authors research efforts in MSI is from Cotton Incorporated (Agreement #08-395) and U.S. Department of Energy, Office of Science, Basic Energy Sciences program (DE-FG02-14ER15647) to KDC. YL was supported by the US Department of Energy, Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences, and Biosciences. The Ames Laboratory is operated by Iowa State University under Contract DE-AC02-07CH11358. We thank Maria Dueñas for obtaining MS images of Arabidopsis seed cross-sections shown in Figure 1.

References:

1. Kueger S, Steinhauser D, Willmitzer L, Giavalisco P: **High-resolution plant metabolomics: from mass spectral features to metabolites and from whole-cell analysis to subcellular metabolite**

distributions. *Plant J* 2012, **70**:39-50.
2. Caprioli RM, Farmer TB, Gile J: **Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS**. *Anal Chem* 1997, **69**:4751-4760.
3. Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM: **Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues**. *Nat Med* 2001, **7**:493-496.

4. Horn PJ, Korte AR, Neogi PB, Love E, Fuchs J, Strupat K, Borisjuk L, Shulaev V, Lee YJ, Chapman KD: **Spatial mapping of lipids at cellular resolution in embryos of cotton**. *Plant Cell* 2012, **24**:622-636.
*5. Horn PJ, Silva JE, Anderson D, Fuchs J, Borisjuk L, Nazarenus TJ, Shulaev V, Cahoon EB, Chapman KD: **Imaging heterogeneity of membrane and storage lipids in transgenic Camelina sativa seeds with altered fatty acid profiles**. *Plant J* 2013, **76**:138-150.
6. Horn PJ, Chapman KD: **Lipidomics in situ: insights into plant lipid metabolism from high resolution spatial maps of metabolites**. *Prog Lipid Res* 2014, **54**:32-52.
7. Horn PJ, Sturtevant D, Chapman KD: **Modified oleic cottonseeds show altered content, composition and tissue-specific distribution of triacylglycerol molecular species**. *Biochimie* 2014, **96**:28-36.
8. Bencivenni M, Faccini A, Zecchi R, Boscaro F, Moneti G, Dossena A, Sforza S: **Electrospray MS and MALDI imaging show that non-specific lipid-transfer proteins (LTPs) in tomato are present as several isoforms and are concentrated in seeds**. *J Mass Spectrom* 2014, **49**:1264-1271.
9. Marques JV, Dalisay DS, Yang H, Lee C, Davin LB, Lewis NG: **A multi-omics strategy resolves the elusive nature of alkaloids in Podophyllum species**. *Mol Biosyst* 2014, **10**:2838-2849.
10. Gorzolka K, Bednarz H, Niehaus K: **Detection and localization of novel hordatine-like compounds and glycosylated derivates of hordatines by imaging mass spectrometry of barley seeds**. *Planta* 2014, **239**:1321-1335.
*11. Dalisay DS, Kim KW, Lee C, Yang H, Rübel O, Bowen BP, Davin LB, Lewis NG: **Dirigent Protein-Mediated Lignan and Cyanogenic Glucoside Formation in Flax Seed: Integrated Omics and MALDI Mass Spectrometry Imaging**. *J Nat Prod* 2015, **78**:1231-1242.
12. Shroff R, Schramm K, Jeschke V, Nemes P, Vertes A, Gershenzon J, Svatoš A: **Quantification of plant surface metabolites by matrix-assisted laser desorption-ionization mass spectrometry imaging: glucosinolates on Arabidopsis thaliana leaves. *Plant J* 2015, **81**:961-972.
13. Abdalsamee MK, Giampà M, Niehaus K, Müller C: **Rapid incorporation of glucosinolates as a strategy used by a herbivore to prevent activation by myrosinases**. *Insect Biochem Mol Biol* 2014, **52**:115-123.
14. Seaman C, Flinders B, Eijkel G, Heeren RM, Bricklebank N, Clench MR: **"Afterlife experiment": use of MALDI-MS and SIMS imaging for the study of the nitrogen cycle within plants. *Anal Chem* 2014, **86**:10071-10077.
15. Klein AT, Yagnik GB, Hohenstein JD, Ji Z, Zi J, Reichert MD, MacIntosh GC, Yang B, Peters RJ, Vela J, et al.: **Investigation of the Chemical Interface in the Soybean-Aphid and Rice-Bacteria Interactions Using MALDI-Mass Spectrometry Imaging**. *Anal Chem* 2015, **87**:5294-5301.
16. Gemperline E, Jayaraman D, Maeda J, Ané JM, Li L: **Multifaceted investigation of metabolites during nitrogen fixation in Medicago via high resolution MALDI-MS imaging and ESI-MS**. *J Am Soc Mass Spectrom* 2015, **26**:149-158.
17. Korte AR, Lee YJ: **MALDI-MS analysis and imaging of small molecule metabolites with 1,5-diaminonaphthalene (DAN)**. *J Mass Spectrom* 2014, **49**:737-741.
*18. Korte AR, Yandeau-Nelson MD, Nikolau BJ, Lee YJ: **Subcellular-level resolution MALDI-MS imaging of maize leaf metabolites by MALDI-linear ion trap-Orbitrap mass spectrometer**. *Anal Bioanal Chem* 2015, **407**:2301-2309.
19. Soltwisch J, Kettling H, Vens-Cappell S, Wiegelmann M, Müthing J, Dreisewerd K: **Mass spectrometry imaging with laser-induced postionization. *Science* 2015, **348**:211-215.

20. Thomas A, Chaurand P: **Advances in tissue section preparation for MALDI imaging MS**. *Bioanalysis* 2014, **6**:967-982.
21. Robichaud G, Garrard K, Barry J, Muddiman D: **MSiReader: An Open-Source Interface to View and Analyze High Resolving Power MS Imaging Files on Matlab Platform**. *J Am Soc Mass Spectrom* 2013, **24**:718-721.

22. Horn P, Chapman K: **Metabolite Imager: customized spatial analysis of metabolite distributions in mass spectrometry imaging**. *Metabolomics* 2014, **10**:337-348.
23. Rübel O, Greiner A, Cholia S, Louie K, Bethel EW, Northen TR, Bowen BP: **OpenMSI: a high-performance web-based platform for mass spectrometry imaging**. *Anal Chem* 2013, **85**:10354-10361.
24. Svatoš A, Bartels B: **Spatially-resolved in vivo plant metabolomics by laser ablation-based mass spectrometry imaging (MSI) techniques: LDI-MSI and LAESI**. *Frontiers in Plant Science* 2015, **6**.
25. Lee YJ, Perdian DC, Song Z, Yeung ES, Nikolau BJ: **Use of mass spectrometry for imaging metabolites in plants**. *Plant J* 2012, **70**:81-95.
26. Bjarnholt N, Li B, D'Alvise J, Janfelt C: **Mass spectrometry imaging of plant metabolites--principles and possibilities**. *Nat Prod Rep* 2014, **31**:818-837.
27. Hankin JA, Barkley RM, Murphy RC: **Sublimation as a method of matrix application for mass spectrometric imaging**. *J Am Soc Mass Spectrom* 2007, **18**:1646-1652.
28. Gemperline E, Rawson S, Li L: **Optimization and comparison of multiple MALDI matrix application methods for small molecule mass spectrometric imaging**. *Anal Chem* 2014, **86**:10030-10035.
29. Horn PJ, James CN, Gidda SK, Kilaru A, Dyer JM, Mullen RT, Ohlrogge JB, Chapman KD: **Identification of a new class of lipid droplet-associated proteins in plants**. *Plant Physiol* 2013, **162**:1926-1936.
30. Franceschi P, Dong Y, Strupat K, Vrhovsek U, Mattivi F: **Combining intensity correlation analysis and MALDI imaging to study the distribution of flavonols and dihydrochalcones in Golden Delicious apples**. *J Exp Bot* 2012, **63**:1123-1133.
31. Ye H, Gemperline E, Venkateshwaran M, Chen R, Delaux PM, Howes-Podoll M, Ané JM, Li L: **MALDI mass spectrometry-assisted molecular imaging of metabolites during nitrogen fixation in the Medicago truncatula-Sinorhizobium meliloti symbiosis**. *Plant J* 2013, **75**:130-145.
32. Louie KB, Bowen BP, McAlhany S, Huang Y, Price JC, Mao JH, Hellerstein M, Northen TR: **Mass spectrometry imaging for in situ kinetic histochemistry**. *Sci Rep* 2013, **3**:1656.
33. Jun JH, Song Z, Liu Z, Nikolau BJ, Yeung ES, Lee YJ: **High-Spatial and High-Mass Resolution Imaging of Surface Metabolites of Arabidopsis thaliana by Laser Desorption-Ionization Mass Spectrometry Using Colloidal Silver**. *Anal Chem* 2010, **82**:3255-3265.
34. Sarsby J, Towers MW, Stain C, Cramer R, Koroleva OA: **Mass spectrometry imaging of glucosinolates in Arabidopsis flowers and siliques**. *Phytochemistry* 2012, **77**:110-118.
35. Li C, Wang Z, Jones AD: **Chemical imaging of trichome specialized metabolites using contact printing and laser desorption/ionization mass spectrometry**. *Anal Bioanal Chem* 2014, **406**:171-182.
36. Seneviratne HK, Dalisay DS, Kim KW, Moinuddin SG, Yang H, Hartshorn CM, Davin LB, Lewis NG: **Non-host disease resistance response in pea (Pisum sativum) pods: Biochemical function of DRR206 and phytoalexin pathway localization**. *Phytochemistry* 2015, **113**:140-148.
37. Debois D, Jourdan E, Smargiasso N, Thonart P, De Pauw E, Ongena M: **Spatiotemporal monitoring of the antibiome secreted by Bacillus biofilms on plant roots using MALDI mass spectrometry imaging**. *Anal Chem* 2014, **86**:4431-4438.
38. Rompp A, Guenther S, Schober Y, Schulz O, Takats Z, Kummer W, Spengler B: **Histology by mass spectrometry: label-free tissue characterization obtained from high-accuracy bioanalytical imaging**. *Angew Chem Int Ed Engl* 2010, **49**:3834-3838.
39. Zavalin A, Yang J, Caprioli R: **Laser beam filtration for high spatial resolution MALDI imaging mass**

spectrometry. *J Am Soc Mass Spectrom* 2013, **24**:1153-1156.
40. Zavalin A, Yang J, Hayden K, Vestal M, Caprioli RM: **Tissue protein imaging at 1 mum laser spot diameter for high spatial resolution and high imaging speed using transmission geometry MALDI TOF MS**. *Anal Bioanal Chem* 2015, **407**:2337-2342.

41. Ellis SR, Bruinen AL, Heeren RM: **A critical evaluation of the current state-of-the-art in quantitative imaging mass spectrometry**. *Anal Bioanal Chem* 2014, **406**:1275-1289.
42. Soares MS, da Silva DF, Forim MR, da Silva MF, Fernandes JB, Vieira PC, Silva DB, Lopes NP, de Carvalho SA, de Souza AA, et al.: **Quantification and localization of hesperidin and rutin in Citrus sinensis grafted on C. limonia after Xylella fastidiosa infection by HPLC-UV and MALDI imaging mass spectrometry**. *Phytochemistry* 2015, **115**:161-170.
43. Zaima N, Yoshimura Y, Kawamura Y, Moriyama T: **Distribution of lysophosphatidylcholine in the endosperm of Oryza sativa rice**. *Rapid Commun Mass Spectrom* 2014, **28**:1515-1520.
44. Kusari S, Lamshöft M, Kusari P, Gottfried S, Zühlke S, Louven K, Hentschel U, Kayser O, Spiteller M: **Endophytes are hidden producers of maytansine in Putterlickia roots**. *J Nat Prod* 2014, **77**:2577-2584.
45. Gemperline E, Li L: **MALDI-mass spectrometric imaging for the investigation of metabolites in Medicago truncatula root nodules**. *J Vis Exp* 2014.
46. Berisha A, Dold S, Guenther S, Desbenoit N, Takats Z, Spengler B, Römpp A: **A comprehensive high-resolution mass spectrometry approach for characterization of metabolites by combination of ambient ionization, chromatography and imaging methods**. *Rapid Commun Mass Spectrom* 2014, **28**:1779-1791.
47. Araújo P, Ferreira MS, de Oliveira DN, Pereira L, Sawaya AC, Catharino RR, Mazzafera P: **Mass spectrometry imaging: an expeditious and powerful technique for fast in situ lignin assessment in Eucalyptus**. *Anal Chem* 2014, **86**:3415-3419.
48. Vanhercke T, El Tahchy A, Liu Q, Zhou XR, Shrestha P, Divi UK, Ral JP, Mansour MP, Nichols PD, James CN, et al.: **Metabolic engineering of biomass for high energy density: oilseed-like triacylglycerol yields from plant leaves**. *Plant Biotechnol J* 2014, **12**:231-239.
49. Yoshimura Y, Zaima N, Moriyama T, Kawamura Y: **Different localization patterns of anthocyanin species in the pericarp of black rice revealed by imaging mass spectrometry**. *PLoS One* 2012, **7**:e31285.
50. Yoshimura Y, Enomoto H, Moriyama T, Kawamura Y, Setou M, Zaima N: **Visualization of anthocyanin species in rabbiteye blueberry Vaccinium ashei by matrix-assisted laser desorption/ionization imaging mass spectrometry**. *Anal Bioanal Chem* 2012, **403**:1885-1895.
51. Hölscher D, Fuchser J, Knop K, Menezes RC, Buerkert A, Svatoš A, Schubert US, Schneider B: **High resolution mass spectrometry imaging reveals the occurrence of phenylphenalenone-type compounds in red paracytic stomata and red epidermis tissue of Musa acuminata ssp. zebrina cv. 'Rowe Red'**. *Phytochemistry* 2015, **116**:239-245.
52. Veličković D, Herdier H, Philippe G, Marion D, Rogniaux H, Bakan B: **Matrix-assisted laser desorption/ionization mass spectrometry imaging: a powerful tool for probing the molecular topology of plant cutin polymer**. *Plant J* 2014, **80**:926-935.

Reference Annotations:

*[5]-Using cryosections of RNAi *Camelina sativa* seeds, the authors use MALDI-MSI to demonstrate that metabolite distributions need to be considered when developing genetic

engineering strategies for optimal production desired compounds.

*[11]-Here transcriptomics, genomics, metabolomics, and MALDI-MSI are used to study the synthesis and distribution of polymeric lignans and cyanogenic glucosides. It was discovered that dirigent proteins are involved with the spatiotemoral formation of metabolites in these pathways, and were found to be differentally localized in flax capsules.

**[12]- MALDI-MSI was used to quantify and genereate MS images of glucosinolates directly off the adaxial and abaxial surfaces of *Arabidopsis thaliana* leaves. These efforts were validated using LAESI-MSI and LESA-MSI and techniques demostrated here could be applied to other plant surface metabolies.

**[14]- MALDI-MSI and SIMS are used to track plant uptake of nutrients containing isotopically labeled ^{15}N. MS images of radish leaves and bulb cryo-sections showed the incorporation of ^{15}N into phosphocholine and choline.

*[18]-A Thermo MALDI-LTQ-Orbitrap Discovery was modified with a Nd:YAG laser capable of ~9 µm laser spot size. Examining cryo-sections of juvenile *Zea mays* leaves, this laser set up and oversampling at 5 µm were used to examine the subcellular metabolite distributions in the upper and lower epidermis, bundle sheath, mesophyll, and vasculature.

**[19]-The authors developed a MALDI instrument with a post ionization source and 5 µm laser spot size. The secondary ionization provided by this instrument increased sensitivity by up to a remarkable two orders of magnitude and was used to generate MS images for a variety of different molecules in positive and negative mode, and in plant and animal tissues.

Figure Legends:

Figure 1. Typical Workflow of a MALDI-MSI Experiment

a-b) Cryo-sectioned tissue(s) (shown-*Arabidopsis thaliana* silique) are coated with matrix by spraying or sublimation. **c-d)** A laser beam is irradiated over the tissue and a mass spectrum is

recorded at each x and y position. **e)** False colored chemical images are generated for ion(s) of interest. **f)** Identities of selected ion(s) are validated by MS/MS or other methods.

Figure 2. Overlaid MALDI-MS Images of a Few Compounds in a Juvenile Maize Cross-Section Obtained with 5 µm Spatial Resolution

a) Optical image, **b)** flavonoids in upper and lower epidermal cell layers, showing rutin and maysin almost exclusively localized in a few upper epidermal cells, **c)** PG(34:2) localized in bundlesheath and SQDG in both bundlesheath and mesophyll, resulting in bundlesheath cells in purple, **d)** non-overlapping images of HMBOA-Glc and DIMBOA-Glc in vacuoles and SQDG in chloroplast. Reproduced from reference [18] with permission.

Table 1. Organisms, Tissues, Target Molecules and Matrices for MALDI-MSI in Plant Tissue Sections and on Tissue Surfaces

Organism	Year	Tissue Type	Imaged Molecules	Matrix Used* (ion mode)	Reference
In Tissue Sections					
Linum usitatissimum; Flax	2015	Seed Capsule	Cyanogenic Glucosides	DHB (+)	[11]
Citrus sinensis; Rangpur lime	2015	Vasculature	Hesperidin, Rutin	CHCA (+), DHB (+)	[42]
Braeburn apple	2015	Hypanthium, skin	Dihexose, Glycoside quercitin pentoside	Norharmane (-)	[19]
Pisum sativum; Pea	2015	Pea Pods	Phytoalexins	DHB (+)	[36]
Zea mays; Corn	2015	Leaves	Amino acids, Organic acids, Glycerolipids, Flavone aglycones	DAN (-)	[18]
Podophyllum hexandrum, P. peltatum; Himalayan Mayapple	2014	Roots	Aporphine alkaloids	DHB (+)	[9]
Hordeum vulgare; Barley	2014	Seed	Hordatines	DHB (+)	[10]
Oryza sativa; Rice	2014	Seed	Lysophosphatidylcholine	DHB (+)	[43]
Putterlickia verrucosa, P. retrospinosa;	2014	Roots	Maytansine	DHB (+)	[44]
Medicago truncatula	2014	Nodules	Organic acids, Amino acids	DHB (+)	[45]
Raphanus sativus; Radish	2014	Leaf and Bulb	Choline, Phosphocholine	CHCA (+), DHB (+)	[14]
Zea mays; Corn	2014	Leaves	Amino acids, Organic acids, Flavonoids, Sulfolipids	DAN (-), 9-AA (-)	[17]
Accent grape	2014	Berries	Anthocyanins, Phospholipids, Triacylglycerols	DHB (+)	[46]
Tomato	2014	Fruits	Lipid transfer protein	CHCA (+)	[8]
Medicago truncatula	2013	Nodules, Roots	Organic acids, Amino acids,	DHB (+), DMAN (-)	[31]

Arabidopsis thaliana	2013	Seeds	Sugars, Lipids, Flavonoids Triacylglycerols, Phosphatidylcholine	DHB (+)	[6]
Gossypium hirsutum; Cotton	2013	Embryos	Triacylglycerols	DHB (+)	[7]

Eucalyptus globulus, E. grandis; Eucalyptus	2013	Stem	Lignin	Silica	[47]
Nicotiana tabacum; Tobacco	2013	Leaves	Triacylglycerols	DHB (+)	[48]
Camelina sativa	2013	Seeds	Triacylglycerols, Phosphatidylcholine	DHB (+)	[5]
Persea americana; Haas Avocado	2013	Mesocarp	Triacylglycerols	DHB (+)	[29]
Oryza sativa; Rice	2012	Seed	Anthocyanins	DHB (+)	[49]
Gossypium hirsutum; Cotton	2012	Embryos	Triacylglycerols, Phospholipids	DHB (+)	[4]
Vaccinium ashei; Blueberry	2012	Berries	Anthocyanins	DHB (+)	[50]
Golden Delicious Apple	2011	Hypanthium, peel, pericarp	Flavonols, Dihydrochalcones	CHCA (-)	[30]
On Tissue Surfaces					
Arabidopsis thaliana	2015	Leaves	Glucosinolates	9-AA (-)	[12]
Musa acuminata; Banana	2015	Stem Epidermis, Stomata	Phenylphenalenones	Matrix Free	[51]
Glycine max (Soybean); Oryza sativa (Rice)	2015	Leaves	Phytoalexins, momilactones, phytocassanes, Salicylic acid, Isoflavones	DAN (-), Fe3O4 (+), TiO2 (+)	[15]
Nectarine, Gala/Dorian/Gold Kiss apple, Tomato	2014	Cutin	Polymers	DHB (+)	[52]
Solanum lycopersicum (Tomato); Tobacco; *Arabidopsis thaliana*	2014	Roots	Lipopeptides, Surfactins	CHCA (+)	[37]
Solanum habrochaites	2013	Trichomes	Acyl Sugars, Flavonoids, Alkaloids, Sesquiterpene Acids	Matrix Free	[35]
Arabidopsis thaliana	2012	Flowers, Siliques	Glucosinolates	9-AA (-)	[34]

* (+) and (-) indicates whether the matrix was used in positive or negative detection mode
Abbreviations: DHB-2,5 dihydroxybenzoic acid, **CHCA**-α-cyano-4-hydroxycinnamic acid, **9-AA**-9 aminoacridine, **DAN**-1,5 diaminonapthalene, **DMAN**- 1, 8 bis(dibethyl-amino)naphthalene.

Figure 1

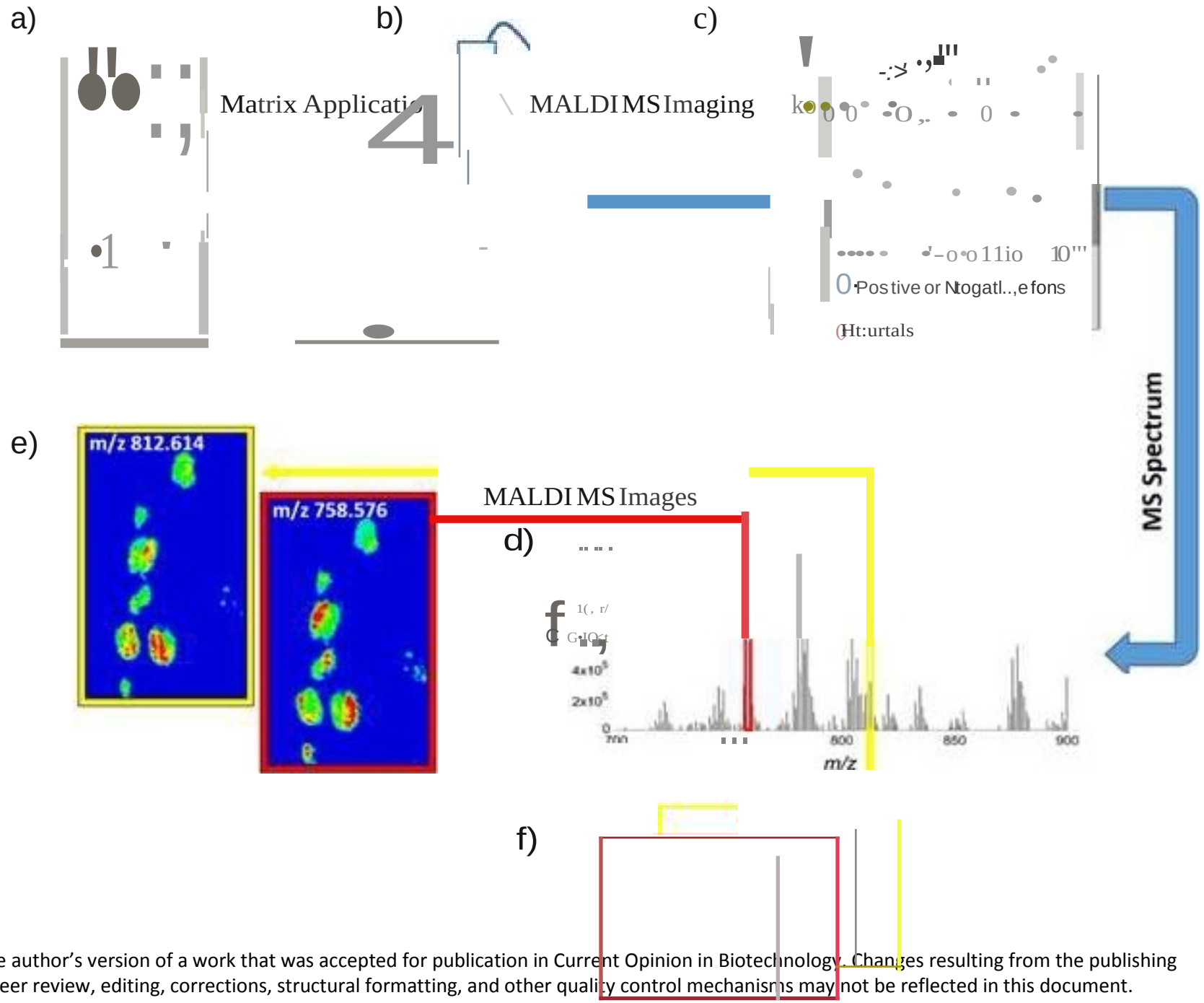

Figure 2

MS/MS" Validation

Figure 3