Phytochemical map leads to local omics world

Ryo Nakabayashi

RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
Author for correspondence: R. Nakabayashi, ryo.nakabayashi@riken.jp

Summary: Imaging mass spectrometry (IMS) is an analytical method for visualizing the localization of metabolites. IMS analysis involves performing spatial mass spectrometry in coordinates specified on cross-section or longitudinal section of organisms, including plants. The localization of detected metabolites can be visualized using the m/z value and signal intensity acquired in IMS analysis. The development of genome or transcriptome sequencing technologies enables us to perform extremely local part analyses at the level of the cells, tissues, or organs. Using the sequencing technologies, phytochemical genomics researches have shown that specialized metabolic pathways have associations between gene expression and metabolite accumulation in a tissue- or organ-specific manner. These findings suggest that the identification of specialized metabolites in the local parts through IMS analysis makes narrowing down biosynthetic genes more simplified. The IMS analysis is potentially capable of increasing the efficiency and accuracy in phytochemical genomics. Herein, I provide recent updates on IMS analysis in plants.

Key words: imaging mass spectrometry, metabolomics, phytochemical genomics, specialized metabolite

INTRODUCTION

Mass spectrometry (MS) is one of the most frequently used methods for the analysis of metabolites in metabolomics (Tsugawa 2018, Wolfender et al. 2019). MS analysis enables us to acquire structural information, such as m/z value, signal intensity, and isotopic pattern of precursor ions derived from metabolites with high sensitivity and coverage. In tandem MS (MS/MS) analysis, more structural information, including substructures, can be acquired from product ions and neutral losses, which are fragments of the precursor ions. For reducing the effect of ion suppression and acquiring better quality data from as many metabolites as possible, another method is used for separating the metabolites before MS or MS/MS analysis. The selected method varies according to the physicochemical properties of target metabolites; for instance, gas chromatography is used for volatile and derivatized primary metabolites, capillary electrophoresis is used for ionic metabolites, and liquid chromatography (LC) is used for lipids and specialized metabolites (Saito and Matsuda 2010, Lei et al. 2011). In LC-MS or LC-MS/MS analysis, retention time can be used for estimating the physicochemical properties as well as the above-described data in MS or MS/MS analysis. Polyhedral data of a metabolite is useful for its chemical assignment.

Imaging mass spectrometry (IMS) is an analytical method for visualizing the localization of metabolites by adding spatial data to MS data in cross or longitudinal section of organisms (Buchberger et al. 2018). Several types of MS can be used for IMS analysis; these include secondary ion, desorption electrospray ionization, laser ablation electrospray ionization, and matrix-assisted laser desorption ionization (MALDI) (Lee et al. 2012, Addie et al. 2015, Etalo et al. 2015). Of them MALDI is widely used in IMS analysis (Fujimura and Miura 2014, Li et al. 2014, Yamamoto et al. 2016, Baker et al. 2017, Duenas et al. 2017, Kompauer et al. 2017, 2017, Shariatgorji et al. 2019, Yamamoto et al. 2019). Along with the section of the organisms, MALDI-IMS analysis requires a matrix reagent for helping to

ionize metabolites on the surface of the section. Spraying the matrix reagent dissolved in an organic solvent onto the section and drying the solvent multiple times helps to extract metabolites on the surface and crystallize the matrix with the metabolites. MS analysis according to the coordinates in the section provides spatial data. Spatial MS analyzes the matrix-sprayed section via laser pulse. The range of laser pulses can be set in an arbitrary manner. The localization of detected metabolites is visualized using the signal intensities of ions on the coordinates (**Figure 1**).

Phytochemical genomics is the study of the identification of genes involved in biosynthetic pathways of primary metabolites and specialized metabolites in plants (Saito 2013). Thus far, many of the biosynthetic genes have been identified in the pathways of specialized metabolites (Fernie and Tohge 2017, Alseekh and Fernie 2018). Multi-omics of genomics/transcriptomics and metabolomics analyses have shown associations between expression of genes and accumulation of metabolites in a tissue- or organ-specific manner (Yonekura-Sakakibara et al. 2008, Suzuki et al. 2015, Li et al. 2016, Tohge et al. 2016, Colinas and Goossens 2018). The manner suggests that “cobehavior” analysis using transcriptome and metabolome data in samples more accumulating arbitrary metabolites and less makes narrowing down candidate genes easier (Rai et al. 2017). Recently, genome or transcriptome sequencing technologies have achieved remarkable breakthroughs. These technologies provide us with a chance to perform multi-omics analysis at the level of the cells, tissues, or organs. It is important to obtain data from only target parts, but not from extraneous parts in the spacial analyses. Reducing contamination increase the quality of data in the target parts (**Figure 2**).

It is expected that combining IMS analysis with other -omics study increases the efficiency and accuracy of narrowing down candidate genes in phytochemical genomics in a local manner by identifying the localization of target metabolites. Due to the mechanical issues such as the limitation of spatial resolution by laser, performing MALDI-IMS analysis is challenging at the

single-cell or organelle level. However, recent approaches have shown that this method can be used for metabolome analysis at the tissue- or organ-level. Herein, I reviewed recent updates on the use of MALDI-IM analysis in plants. The general principles,

experimental procedures, and examples of studies that utilize this technique have been well-described in previous reviews (Boughton et al. 2016, Dong et al. 2016).

Figure 1. Overview of matrix-assisted laser desorption ionization-imaging mass spectrometry.

Figure 2. Strategy of multi-omics analysis using imaging mass spectrometry.

WORKFLOW OF MALDI-IMS ANALYSIS

Generally, MALDI-IMS analysis consists of the following seven steps: 1) estimation of data size, 2) image of figure, 3) preparation of section, 4) selection of matrix reagent, 5) spraying the reagent, 6) data acquisition, and 7) data analysis. Understanding every single step is necessary to maximize reproducibility and to produce good quality data.

ESTIMATION OF DATA SIZE

Understanding the size of dataset is important for handling IMS platform. Previous studies using ultrahigh-resolution MALDI-IMS analysis provided raw data at the gigabyte scale during each run (Nakabayashi et al. 2017, 2019). Although it is expected that other instruments of IMS analysis may provide less data, a high-spec computational environment is required for data acquisition, storage, and processing of IMS data.

IMAGE OF FIGURE

Images of figures for publication need to be considered before the analysis. By considering other data, it can be decided which cross or longitudinal section is more appropriate. Since limited space is available on a glass slide, bigger leaves may often not fit on the slide. However, if the leaf is rolled, IMS analysis can be performed on its cross or longitudinal section. For instance, MALDI-IMS analysis was used for analyzing a cross-section of a rolled leaf from *Catharanthus roseus*, a monoterpene indole alkaloid (MIA)-producing medicinal plant (O'Connor and Maresh 2006). The IMS analysis showed the localization of MIAs in the leaf tissues (Nakabayashi et al. 2017).

Labeling the stable isotopes produces a mass shift of m/z values between stable isotope-labeled and non-labeled metabolites in MS and MS/MS analysis (Giavalisco et al. 2011, Nakabayashi et al. 2013, Glaser et al. 2014, Wang and Jones 2014, Nakabayashi et al. 2015, Nakabayashi et al. 2016, Freund and Hegeman 2017, Kera et al. 2018, Hautbergue et al. 2019, Tsugawa et al. 2019). It is expected that stable isotope- and non-labeled metabolites can be mainly detected in stable isotope- and non-labeled plants, respectively. Stable isotope-labeled and non-labeled metabolites can not be detected in the non-labeled and stable isotope-labeled plants, respectively. Comparative analyses showed that the mirror image of stable isotope-labeled and non-labeled metabolites can not be obtained (Nakabayashi et al. 2017).

PREPARATION OF SECTION

Fresh plant samples are often required for IMS analysis. Therefore, for analyzing transformants, mutants, or harmful plants, the administration must allow the importation of these plants into the laboratory.

Fresh samples are frozen to quench enzymatic and metabolic activities, and their shapes are maintained using embedding reagent. Using a block of the reagent-embedded sample, one or a few sections are cut and placed on a glass slide and freeze-dried. The freeze-drying process often results in peeling or warpage due to the removal of water content from the

section or another mechanical reason. Moreover, laser pulses may cause deformation of the section. Therefore, MALDI-IMS analysis requires that section withstands to thousands or tens of thousands of laser pulses. For that the section needs to tightly adhere to the slide. Recently, a technique called the NakaMi method has been developed for helping the section to adhere to the slide using transfer tape and conductive double-sided tape (Figure 3) (Nakabayashi et al. 2019). This method can be used for preparing sections of fruits, flowers, leaves, petioles, stems, and roots.

Figure 3. Overview of NakaMi method.

SELECTION OF MATRIX REAGENT

Several matrix reagents may be used for visualizing the localization of phytochemicals; primary metabolites (carbohydrates, lipids, and fatty acids); and specialized metabolites (alkaloids, glucosinolates, and flavonoids) (Boughton et al. 2016, Dong et al. 2016). For analyzing a

single metabolite or a metabolite group, the reported reagents are sufficient. However, there is limited information available regarding which matrix reagent should be selected for analyzing a wide range of specialized metabolites. A previous study used α -cyano-4-hydroxycinnamic acid (CHCA), 1,5-diaminonaphthalene (DAN), 2,5-dihydroxybenzoic acid (DHB), and 3-hydroxypicolinic acid (HPA) as matrix reagents to analyze 36 metabolites belonging to 12 groups (Nakabayashi et al. 2019). The metabolites were analyzed under the same analytical conditions (Table 1). The results suggested that both CHCA and DHB are appropriate for detecting a wide range of metabolites in positive ion mode. The coverage of the specialized metabolites indicated that MALDI-IMS analysis can be used for metabolomics.

SPRAYING THE MATRIX REAGENT

As described above, spraying the matrix reagent is essential for obtaining mixed crystals containing the matrix and extracted metabolites. Spraying the matrix reagent in excess results in ion suppression due to the high amount of the matrix. Meanwhile, spraying insufficient matrix reagent results in poor detection of metabolites due to the low quantity of extracted metabolites. A small crystal size results in high-quality spatial data. For obtaining good quality data, the matrix reagent must be uniformly sprayed onto the section multiple times to prepare fine crystals. Manual spraying may be performed using an airbrush, but this method requires a high skill level. Alternatively, an automatic sprayer may be used for creating uniform crystals. There are several commercial sprayers available; the appropriate sprayer must be selected on the basis of the advantages; it provides in terms of spray, extraction, time, and warming.

DATA ACQUISITION

Before initiating the analysis, the range of laser pulses should be set according to the signal intensity of ions derived from the metabolites. When metabolites are easily ionized and provide high signal intensity, the range of laser pulses can be set to small (<50 μm). However, when metabolites are not easily ionized, the range must be set to large (>100 μm). The time of analysis depends on the data points and the number of pulses. It may take several hours to acquire all the data.

DATA ANALYSIS

For understanding the localization of metabolites, tens of thousands of data points must be processed. Existing analytical approaches and software tools enable us to routinely identify the general or specific localization of metabolites, including specialized metabolites (Nakabayashi et al. 2019). Segmentation analysis using the vendor software SCiLS Lab (Bruker Daltonics, Germany) provides a top-down clustering using bisecting k -means that allows for the arbitrary segmentation of metabolite features in a reasonable time frame. The output figure is a map that reflects the localization of analyzed metabolites using color labeling. The localization of metabolites in the spear and root of asparagus (*Asparagus officinalis*) is identified

using this method (Figure 4). This method is helpful for a quick understanding of tissue-redundant metabolites.

Figure 4. Segmentation analysis in asparagus (*Asparagus officinalis*). The bisecting k -means analysis provides patterns of the accumulation of detected metabolites. Each color indicates a pattern meaning that metabolites accumulate more at the part than the others. Upper, spear; lower, root.

FUTURE PERSPECTIVE

MALDI-IMS is a non-separation analytical method that results in ion suppression because hundreds of metabolites are probably analyzed at a single data point. This is a limitation of acquiring MS/MS data in sections. For analyzing the metabolites at a higher level of sensitivity, this method needs to be combined with a separation technique. Introducing ion mobility (IM) to MS or MS/MS analysis enables us to separate metabolites, increase detected metabolites, and improve the quality of data. This method is also capable of separating isobaric structural isomers and analogs (Schroeder et al. 2019). Plants produce structural isomers and analogs. It is, therefore, expected that IM-MALDI-IMS analysis (Spraggins et al. 2019) provides new insights into the role of the structural isomers/analogues on specialized metabolites.

CONCLUSION

Here, I have summarized the recent updates on MALDI-IMS analysis and its potential capacity for enhancing researches in phytochemical genomics. For identifying the function of genes, multiomics analysis of transcriptomics and metabolomics analysis is performed. Comparative analysis using transformants/mutants has allowed for the identification of the function of metabolites (Nakabayashi et al. 2014, Nakabayashi and Saito 2015, Massalha et al. 2017, Huang et al. 2019). As described at the beginning of this review, polyhedral data is important for understanding the metabolite. The addition of spatial data to MS data provides insights regarding the biological and physiological functions of metabolites. Plant specialized metabolites may help mitigate environmental stress such as biotic and abiotic

Table 1. Screening of matrix reagent using specialized metabolites.

type	name	molecular formula	adduct type	exact mass	CHCA	DAN	DHB	HPA
alkaloid	eserine	C ₁₅ H ₂₁ N ₃ O ₂	[M+H] ⁺	276.170653	++	+	+++	++++
			[M+Na] ⁺	298.152598	+++	N.D.	++++	++
	kopsinine	C ₂₁ H ₂₆ N ₂ O ₂	[M+H] ⁺	339.206705	+++	N.D.	++++	++
			[M+Na] ⁺	361.188649	++++	N.D.	+++	N.D.
	voacamine	C ₄₃ H ₅₂ N ₄ O ₅	[M+H] ⁺	705.401047	++	+	++++	N.D.
			[M+Na] ⁺	727.382991	++	+++	N.D.	++++
anthocyanidin	cyanidin	C ₁₅ H ₁₁ O ₆	M ⁺	287.055014	+++	++	++++	N.D.
			[M+Na] ⁺	310.044784	N.D.	N.D.	N.D.	N.D.
	delphinidin	C ₁₅ H ₁₁ O ₇	M ⁺	303.049929	++++	N.D.	+++	N.D.
			[M+Na] ⁺	326.039698	N.D.	N.D.	N.D.	N.D.
	malvidin	C ₁₇ H ₁₅ O ₇	M ⁺	331.081229	+++	N.D.	++++	++
			[M+Na] ⁺	354.070999	N.D.	N.D.	N.D.	N.D.
anthocyanin	cyanidin 3,5-di- <i>O</i> -glucoside	C ₂₇ H ₃₁ O ₁₆	M ⁺	611.160661	++++	+	+++	++
			[M+Na] ⁺	634.150431	++++	N.D.	+++	++
	malvidin 3,5- <i>O</i> -diglucoside	C ₂₉ H ₃₅ O ₁₇	M ⁺	655.186876	++++	+	+++	++
			[M+Na] ⁺	678.176645	+++	N.D.	++++	N.D.
	peonidin 3,5- <i>O</i> -di-glucopyranoside	C ₂₈ H ₃₃ O ₁₆	M ⁺	625.176311	+++	+	++++	++
			[M+Na] ⁺	648.166081	N.D.	N.D.	++++	N.D.
flavonoid aglycone	apigenin	C ₁₅ H ₁₀ O ₅	[M+H] ⁺	271.060100	++++	+	+++	++
			[M+Na] ⁺	293.042044	++++	N.D.	+++	++
	kaempferol	C ₁₅ H ₁₀ O ₆	[M+H] ⁺	287.055014	+++	N.D.	++++	++
			[M+Na] ⁺	309.036959	++++	N.D.	+++	++
	quercetin	C ₁₅ H ₁₀ O ₇	[M+H] ⁺	303.049929	++++	+	+++	++
			[M+Na] ⁺	325.031873	++++	N.D.	+++	++
flavonoid glycoside	homoorientin	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺	449.107838	++++	+	+++	++
			[M+Na] ⁺	471.089782	++++	+	+++	++
	kaempferol 3- <i>O</i> -glucoside	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺	449.107838	+++	N.D.	++	++++
			[M+Na] ⁺	471.089782	++++	+	+++	++
	rutin	C ₂₇ H ₃₀ O ₁₆	[M+H] ⁺	611.160661	++	+	+++	++++
			[M+Na] ⁺	633.142606	++	+	++++	+++
glucosinolate	butenyl glucosinolate	C ₁₁ H ₁₉ NO ₉ S ₃	[M+H] ⁺	406.029471	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	428.011415	N.D.	N.D.	N.D.	N.D.
	4-methylthiobutyl glucosinolate	C ₁₂ H ₂₃ NO ₉ S ₃	[M+H] ⁺	422.060771	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	444.042715	N.D.	N.D.	N.D.	N.D.
	sinigrin	C ₁₀ H ₁₇ NO ₉ S ₂	[M+H] ⁺	360.041750	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	382.023694	N.D.	N.D.	N.D.	N.D.
lignan	lariciresinol	C ₂₀ H ₂₄ O ₆	[M+H] ⁺	361.164565	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	383.146509	++++	N.D.	+++	++
	pinoresinol	C ₂₀ H ₂₂ O ₆	[M+H] ⁺	359.148915	+++	N.D.	++++	N.D.
			[M+Na] ⁺	381.130859	+++	N.D.	++++	N.D.
	secoisolariciresinol diglucoside	C ₃₂ H ₄₆ O ₁₆	[M+H] ⁺	687.285862	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	709.267806	++	+	++++	+++
phenolamide/hydroxycinnamic acid amide	<i>N</i> - <i>p</i> -coumaroyltyramine	C ₁₇ H ₁₇ NO ₃	[M+H] ⁺	284.128120	++++	N.D.	+++	++
			[M+Na] ⁺	306.110064	+++	N.D.	++++	++
	kukoamin A	C ₂₈ H ₄₂ N ₄ O ₆	[M+H] ⁺	531.317712	++	+	++++	+++
			[M+Na] ⁺	553.299656	++	N.D.	++++	+++
	tricoumaroyl spermidine	C ₃₄ H ₃₇ N ₃ O ₆	[M+H] ⁺	584.275512	+++	+	++++	++
			[M+Na] ⁺	606.257457	+++	+	++++	++
phenylpropanoid/coumarin	chlorogenic acid	C ₁₆ H ₁₈ O ₉	[M+H] ⁺	355.102359	N.D.	N.D.	++++	N.D.
			[M+Na] ⁺	377.084303	+++	N.D.	N.D.	++++
	imperatorin	C ₁₆ H ₁₄ O ₄	[M+H] ⁺	271.096485	++++	N.D.	+++	++
			[M+Na] ⁺	293.078430	+++	N.D.	++++	++
	sinapic acid	C ₁₁ H ₁₂ O ₅	[M+H] ⁺	225.075750	++++	+++	N.D.	N.D.
			[M+Na] ⁺	247.057694	N.D.	++++	N.D.	N.D.
saponin/glycoalkaloid aglycone	sarsapogenin	C ₂₇ H ₄₄ O ₃	[M+H] ⁺	417.336322	++	N.D.	++++	+++
			[M+Na] ⁺	439.318266	N.D.	N.D.	++++	N.D.
	soyasapogenol A	C ₃₀ H ₅₀ O ₄	[M+H] ⁺	475.378187	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	497.360131	+++	N.D.	++++	++
	tomatidine	C ₂₇ H ₄₅ NO ₂	[M+H] ⁺	416.352306	+++	++	++++	N.D.
			[M+Na] ⁺	438.334250	++	+	+++	++++
saponin/glycoalkaloid	ginsenoside Ro	C ₄₈ H ₇₆ O ₁₉	[M+H] ⁺	957.505357	N.D.	N.D.	N.D.	N.D.
			[M+Na] ⁺	979.487301	+++	++	++++	+
	soyasaponin Bb	C ₄₈ H ₇₈ O ₁₈	[M+H] ⁺	943.526092	+	++	+++	++++
			[M+Na] ⁺	965.508036	++	+	++++	+++
	tomatine	C ₅₀ H ₈₃ NO ₂₁	[M+H] ⁺	1034.553035	+++	+	++++	++
			[M+Na] ⁺	1056.534979	+++	+	++	++++
sulfur-containing metabolite	g-glutamylcysteine	C ₈ H ₁₄ N ₂ O ₅ S	[M+H] ⁺	251.069619	N.D.	N.D.	++++	N.D.
			[M+Na] ⁺	273.051563	N.D.	N.D.	N.D.	N.D.
	glutathione	C ₁₀ H ₁₇ N ₂ O ₆ S	[M+H] ⁺	308.091083	++	N.D.	++++	+++
			[M+Na] ⁺	330.073027	+++	N.D.	++++	++
	l-isothiocyanato-9-(methylsulfinyl)-nonal	C ₁₁ H ₂₁ NOS ₂	[M+H] ⁺	248.113733	++++	N.D.	+++	++
			[M+Na] ⁺	270.095677	++	N.D.	++++	+++

N.D., not detected.

+, ++, +++, and +++ can be compared among the matrix reagents, not other metabolites.

stress. Plants typically accumulate such metabolites within their outermost layers (e.g., epidermis). Spatial data will be an important key for understanding the function of metabolites. The role of specialized metabolites remains unknown within many tissues. Hopefully, the active participation of IMS analysis in phytochemical genomics will help determine their roles.

ACKNOWLEDGEMENTS

This work was partially supported by JST the Strategic International Collaborative Research Program, MAFF Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, and the Japan Advanced Plant Science Network.

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Received: 24 January 2020 / Accepted: 11 March 2020