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Chapter 21

Profiling the Jasmonic Acid Responses by Nuclear Magnetic Resonance-Based Metabolomics

Hye Kyong Kim, Young Hae Choi, and Robert Verpoorte

Abstract

Metabolomics based on nuclear magnetic resonance (NMR) can be used to monitor the metabolite response of plants to jasmonic acid. Metabolomics experiments consist of three important steps: sample preparation, NMR analysis, and data mining. In sample preparation, a very critical factor is the selection of a proper solvent for plant material extraction because the presence of metabolites will vary according to the solvent used. For NMR analysis, an intermediate-to-polar solvent, such as a mixture of methanol and water, is a good choice. In general, ^{1}H-NMR spectroscopy is the standard method for metabolite profiling, whereas two-dimensional spectroscopy provides more detailed information on the metabolites. Finally, various chemometric methods can be used for data mining. Here, we describe all three steps of metabolomic analysis by means of NMR spectroscopy.

Key words Metabolomics, NMR spectroscopy, Jasmonic acid, Extraction, Metabolite identification, Data mining

1 Introduction

Jasmonic acid (JA) is a well-known plant signaling molecule involved in various physiological functions of plants, particularly in plant defense responses [1]. The signaling of jasmonate has been thoroughly investigated in the past. JA has been shown to act as an elicitor to induce plant secondary metabolism via extensive transcriptional reprogramming [2, 3], activating entire metabolic pathways. Hence, metabolic responses of plants to JA are very complex and diverse.

Recent advances in analytical technology allow us to analyze organisms comprehensively. One such method is metabolomics, a tool that can be used to draw a whole picture of the organismal behavior under certain conditions [4]. Thus, it can be applied to examine the JA response in plants. Mass spectroscopy and nuclear magnetic resonance (NMR) have emerged as the most suitable

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platforms among many analytical techniques. The advantage of mass spectroscopy is its high sensitivity, but its drawback is the limited range of metabolites that can be detected [5]. In contrast, NMR can detect diverse groups of metabolites, although its low sensitivity still remains a major shortcoming. Considering its high reproducibility, simple sample preparation, and short analysis time, NMR is most suited for high-throughput processes. For example, in case of time courses, in which numbers of samples have to be analyzed to follow the dynamics of the plant's responses to JA treatments over time, NMR is the method of choice [6].

In this chapter, we describe a method for metabolite profiling both in intact plants and cell cultures. In general, experiments on metabolite profiling consist of three steps: (1) sample preparation, (2) sample analysis, and (3) data mining. The experiments start with the JA treatment to plants, subsequent harvest, and extraction prior to the NMR measurement. The NMR analysis itself is quite straightforward, because, whereas ^{1}H NMR is the method of choice for standard profiling, other two-dimensional NMR techniques are utilized to identify the metabolites [7, 8]. Diverse chemometric methods can be used for data mining [9, 10], extracting all information from the NMR data set, such as increasing or decreasing levels of metabolites after JA treatment.

2 Materials

2.1 Equipment

1. Glass rods.
2. Growth chamber.
3. Culture flasks.
4. Pestle and mortar.
5. Plastic tubes.
6. Spatula.
7. Freeze drying machine.
8. Vacuum pump.
9. 1.5-mL Eppendorf tubes.
10. Vortex machine.
11. Ultrasonicator.
12. Microcentrifuge.
13. 5-mm NMR tubes.
14. Spectrometer.
15. NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), including software, such as AMIX (Bruker BioSpin) and SIMCA-P (Umetrics AB, Umeå, Sweden).

2.2 Solvents and Solutions

2.2.1 NMR

1. Deuterium oxide (D_2O).
2. 1.0 M NaOD: 1 mL of NaOD (40 % (w/v) in D_2O, corresponding to 10 M) added to 9 mL of D_2O.
3. 90 mM phosphate buffer (pH 6.0): 1.232 g of KH_2PO_4 and 10 mg (0.01 % (w/v) 3-trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TPS) added to 100 mL D_2O; mix them until completely dissolved and adjust pH with 1.0 M NaOD.
4. CH_3OH-*d4*.

2.2.2 JA

1. Mock solution: 20 % (v/v) EtOH.
2. For plant cell cultures, 100 μM MeJA stock solution: 22.4 mg of MeJA (=21.6 μL) dissolved in 1 mL EtOH; 50 μL of this stock solution to be added to 50 mL of culture-containing flasks [11].
3. For intact plants, 5 mM MeJA solution: 11.2 mg (=10.8 μL) of MeJA, dissolved in 20 % EtOH to obtain 10 mL [12].

3 Methods

Carry out all procedures at room temperature, unless otherwise specified.

3.1 JA Treatment

The MeJA solution should be applied topically on the surface of individual leaves.

3.1.1 Intact Plants

1. Spread the MeJA solution evenly on the surface with a glass rod.
2. Spray mock solution (20 % EtOH) on the control plants evenly with a glass rod.
3. Place the plant in the growth room (25 °C, 50 % humidity) (*see* **Note 1**).

3.1.2 Plant Cell Cultures

1. Add 250 μL of the MeJA solution to the culture flasks.
2. Add an equal amount of the mock solution (ethanol) to the controls.
3. Place the flasks in the growth chamber separately.

3.2 Sample Preparation

3.2.1 Intact Plants

1. After treatment, harvest plants at fixed time points (e.g., at 0 h, 12 h, 1 day, 2 days, 3 days, 7 days, and 14 days) and freeze them immediately in liquid nitrogen (*see* **Note 2**).
2. Precool a pestle and mortar with liquid nitrogen.
3. Grind the frozen leaves with the precooled pestle and mortar under liquid nitrogen (*see* **Note 3**).
4. Transfer ground samples to a plastic tube with a spatula.
5. Keep in the deep-freezer before drying.
6. Place frozen samples in the freeze dryer for 1–2 days (*see* **Note 4**).

3.2.2 Plant Cell Cultures

1. Harvest cultures by vacuum filtration.
2. Collect cells with a spatula and transfer to the tubes.
3. Transfer tubes to the freezer at least for 1 day (*see* **Note 2**).
4. Place frozen samples in the freeze dryer for 1–2 days (*see* **Note 4**).

3.3 Sample Extraction

1. Weigh the freeze-dried sample in an Eppendorf tube.
2. Add 0.75 ml of CH_3OH-*d4* and 0.75 ml of KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1 % (w/w) TSP (*see* **Note 5**).
3. Vortex for 1 min.
4. Ultrasonicate for 10–20 min.
5. Centrifuge at room temperature for 5–10 min with a microtube centrifuge (17,000 × *g*) (*see* **Note 6**).
6. Transfer the supernatant (more than 1 mL) to a 1.5-mL Eppendorf tube.
7. If the supernatant is not clear, repeat centrifugation (17,000 × *g* for 1 min at room temperature).
8. Transfer 800 μL of the supernatant to a 5-mm NMR tube.

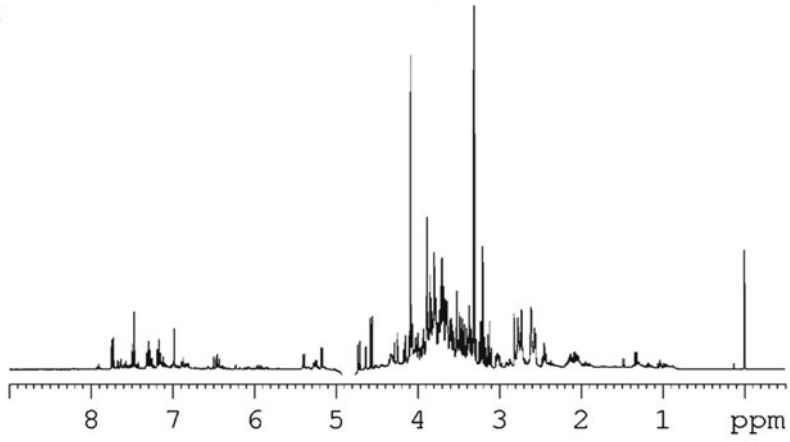
3.4 NMR Measurement

1. Load the NMR tube into the spectrometer.
2. Set the sample temperature at 298 K (25 °C) and leave a few minutes for thermal equilibration.
3. Tune and match the NMR tube.
4. Lock the spectrometer frequency to the deuterium resonance arising from the NMR solvents (either MeOD or D_2O; preferably MeOD).
5. Shim the sample either manually or with an automation system (*see* **Note 7**).
6. Start acquisition for 64 scans (*see* **Note 8**).
7. Set the parameters for standard NMR (*see* **Note 9**).
8. After acquisition, perform Fourier transformation.
9. Correct phase and baseline and calibrate the spectrum by setting the TSP peak at 0.00 ppm or MeOD at 3.3 ppm (Fig. 1a).

3.5 NMR Data Analysis

1. Convert the NMR spectra to a suitable form for further multivariate analysis (Fig. 1b), for instance with the commonly used software AMIX for the conversion to an ASCII file (*see* **Note 10**) (Fig. 1c).
2. Perform a principal component analysis with the SIMCA-P software or an equivalent software (*see* **Note 11**) (Fig. 2).

a



b

Primary ID	Obs. Sec. ID	10.00	9.96	9.92	9.88	9.84	9.80	9.76	9.72
C0-1	C0	0.00085	0	0	0	0	0	0	0.00107
C0-2	C0	0	0	0.01792	0.00709	0.02182	0.00031	0.00099	0.00183
C0-3	C0	0	0	0.00308	0.00283	0.00032	0.02552	0	0.00508
M0-1	M0	0	0	0	0	0	0	0	0
M0-2	M0	0.00778	0	0	0.00195	0.01385	0	0.00721	0.02529
M0-3	M0	0.02844	0.01783	0.04343	0.01691	0.03784	0.0297	0.01503	0.02375
M0-4	M0	0.00092	0.01728	0.01412	0.00514	0.05688	0.00881	0.0345	0.03938
C12-1	C0.5	0	0	0	0	0	0	0	0
C12-2	C0.5	0	0	0	0	0	0	0	0
C12-3	C0.5	0	0	0	0	0	0	0	0
M12-1	M0.5	0.00104	0	0	0	0	0.00064	0	0
M12-2	M0.5	0.00268	0	0	0	0.01143	0.00347	0.00148	0.00762
M12-3	M0.5	0	0	0	0	0	0	0	0
M12-4	M0.5	0.08161	0.1158	0.12959	0.10618	0.0473	0.03822	0.15212	0.24314
C1D-1	C1	0	0	0	0	0	0	0	0

c

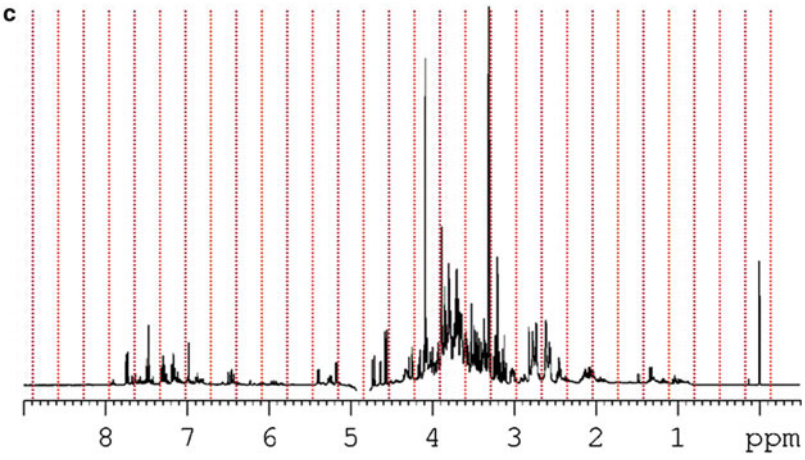


Fig. 1 ^{1}H-NMR spectra and bucketing with small bins (0.04 ppm). (**a**) ^{1}H-NMR spectrum of MeJA-treated *Brassica rapa* (turnip) (adapted from ref. 13 with permission © Elsevier B.V.; http://www.journals.elsevier.com/phytochemistry/). (**b**) Bucketed table with the peak intensity of each bin created with the AMIX software. (**c**) Illustration of bucketing

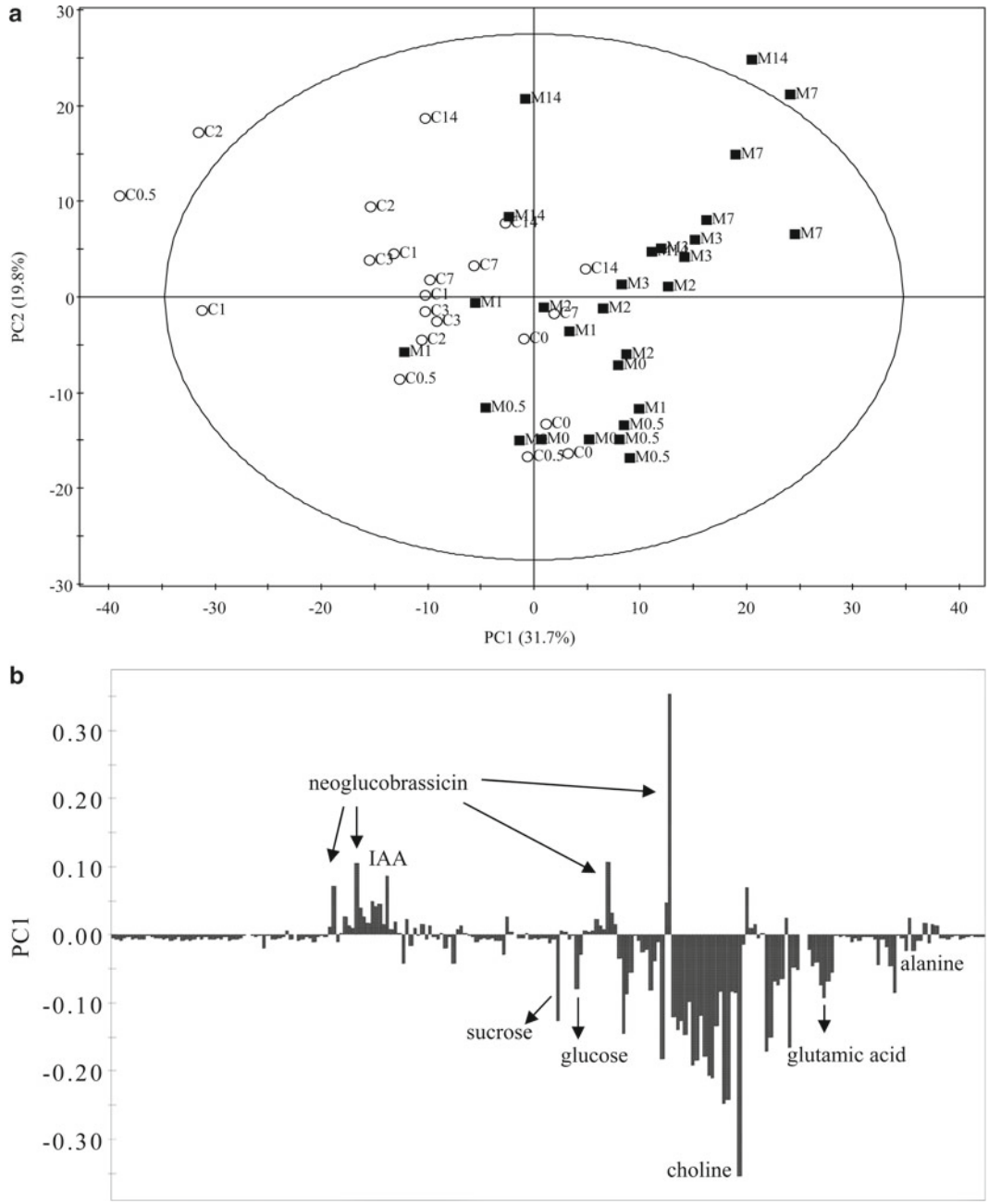


Fig. 2 NMR data from MeJA-treated *Brassica rapa* and corresponding control plants. (**a**) Score plot. (**b**) Loading plot. Several metabolites are indicated that increased with MeJA treatment in *Brassica rapa* (adapted from ref. 13 with permission © Elsevier B.V.; http://www.journals.elsevier.com/phytochemistry/)

4 Notes

1. To avoid the effect of volatile MeJA on control plants, it is recommended to place MeJA-treated plants in a separate growth chamber under conditions identical to those of the control chamber. To ensure reproducible experiments, please keep in mind the various biological variables. Metabolic changes can happen during the day, so the elicitation and harvest times, for instance, should be fixed. Other factors, such as the age of the leaves to be used or the different developmental stages of the plant, will also be reflected in the metabolome. Therefore, they should all be standardized in each experiment.
2. Harvested plant material can be stored at −80 °C for several weeks before extraction.
3. Alternatively, a grinder (ball mill) can be used. However, it is important to always use the same method, because differences can occur depending on the grinding methods.
4. The time required for drying with a freeze dryer can vary according to the machine and environment.
5. The choice of the extraction solvent is very critical, because the type of metabolites extracted depends on it. Intermediate-polar and polar metabolites are well detected by NMR, whereas nonpolar metabolites (fatty acids and terpenoids) are difficult to analyze individually. Although some solvents allow a good differentiation of the metabolites, some others fail to do so, probably not only because of the characteristics of the solvent itself but also because of the possible saturation of metabolites in the extraction solvent. Therefore, we recommend to test various solvents to determine the most suitable conditions for the proposed study. In our protocol, a MeOH–water mixture proved to be the most suitable in terms of the range of covered metabolites [14, 15]. If the full metabolome is to be extracted, a comprehensive extraction method can be considered [16].
6. Variable speeds (14,000–17,000 × *g*) can be used to obtain a clear supernatant.
7. All these steps can be set up in the automation system (for instance, ICON NMR; Bruker BioSpin).
8. The number of scans can vary depending on the concentration of samples. Although 64 scans are the most commonly used, they can be increased to 128 scans. It takes approximately 5–6 min to obtain 64 scans and 10–12 min for 128 scans.
9. Parameters for standard ^{1}H NMR spectroscopy are as follows: Set up pulse sequence comprising relaxation delay-60°-acquire, in which the pulse power is set to achieve a 60° flip angle, 10 kHz spectral width, and water presaturation applied with

1.5-s relaxation delay. Processing parameters are as follows: Zero-fill to 64 k data points with exponential line broadening of 0.3 Hz.

10. Other software, such as MestReNova (Mestrelab Research, Santiago de Compostela, Spain; www.mestrelab.com) and Chenomx (Edmonton, Alberta, Canada; www.chenomx.com), can be used for the file conversion. In this step, the peak is integrated into a small bin (bucket), the size of which is defined by the user. The size is preferably 0.04 ppm to avoid the effect of signal fluctuation due to pH or concentration. The signals of remaining solvents have to be removed for the statistical analysis.
11. For instance, equivalent software can be used as well, such as the Statistical Package for the Social Sciences (SPSS; IBM Corporation, Armonk, NY, USA: http://www-01.ibm.com/software/analytics/spss/), The UnScrambler® (Camo Software AS, Olso, Norway; www.camo.com), and MATLAB (Mathworks, Natick, MA, USA; www.mathworks.com) with statistics toolbox.

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References

1. Farmer EE, Alméras E, Krishnamurthy V (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr Opin Plant Biol 6:372–378
2. De Geyter N, Gholami A, Goormachtig S, Goossens A (2012) Transcriptional machineries in jasmonate-elicited plant secondary metabolism. Trends Plant Sci 17:349–359
3. Pauwels L, Inzé D, Goossens A (2009) Jasmonate-inducible gene: what does it mean? Trends Plant Sci 14:87–91
4. Sumner LW, Mendes P, Dixon RA (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62:817–836
5. Verpoorte R, Choi YH, Mustafa NR, Kim HK (2008) Metabolomics: back to basics. Phytochem Rev 7:525–537
6. Seger C, Sturm S (2007) Analytical aspects of plant metabolite profiling platforms: current standings and future aims. J Proteome Res 6:480–497
7. Colquhoun IJ (2007) Use of NMR for metabolic profiling in plant systems. J Pestic Sci 32:200–212
8. Fan TW-M (1996) Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. Prog Nucl Magn Reson Spectrosc 28:161–219
9. Trygg J, Lundstedt T (2007) Chemometrics techniques for metabonomics. In: Lindon JC, Nicholson JK, Holmes E (eds) The Handbook of Metabonomics and Metabolomics. Elsevier, Amsterdam, pp 171–199
10. van den Berg RA, Hoefsloot HCJ, Westerhuis JA, Smilde AK, van der Werf MJ (2006) Centering, scaling, and transformations:

improving the biological information content of metabolomics data. BMC Genomics 7:142

11. Sánchez-Sampedro A, Kim HK, Choi YH, Verpoorte R, Corchete P (2007) Metabolomic alterations in elicitor treated *Silybum marianum* suspension cultures monitored by nuclear magnetic resonance spectroscopy. J Biotechnol 130:133–142
12. Hendrawati O, Yao Q, Kim HK, Linthorst HJM, Erkelens C, Lefeber AWM, Choi YH, Verpoorte R (2006) Metabolic differentiation of *Arabidopsis* treated with methyl jasmonate using nuclear magnetic resonance spectroscopy. Plant Sci 170:1118–1124
13. Liang Y-S, Choi YH, Kim HK, Linthorst HJM, Verpoorte R (2006) Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by 2-dimensional NMR spectroscopy. Phytochemistry 67:2503–2511
14. Kim HK, Verpoorte R (2010) Sample preparation for plant metabolomics. Phytochem Anal 21:4–13
15. Kim HK, Choi YH, Verpoorte R (2010) NMR-based metabolomic analysis of plants. Nat Protoc 5:536–549
16. Yuliana ND, Khatib A, Verpoorte R, Choi YH (2011) Comprehensive extraction method integrated with NMR metabolomics: a new bioactivity screening method for plants, adenosine A1 receptor binding compounds in *Orthosiphon stamineus* Benth. Anal Chem 83:6902–6906