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NMR-based metabolomic analysis of plants

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Abstract

Nuclear magnetic resonance (NMR)-based metabolomics has many applications in plant science. Metabolomics can be used in functional genomics and to differentiate plants from different origin, or after different treatments. In this protocol, the following steps of plant metabolomics using NMR spectroscopy are described: sample preparation (freeze drying followed by extraction by ultrasonication with 1:1 CD_3OD:KH_2PO_4 buffer in D_2O), NMR analysis (standard ^1H, J-resolved, ^1H–^1H correlation spectroscopy (cosY) and heteronuclear multiple bond correlation (HMBC)) and chemometric methods. The main advantage of NMR metabolomic analysis is the possibility of identifying metabolites by comparing NMR data with references or by structure elucidation using two-dimensional NMR. This protocol is particularly suited for the analysis of secondary metabolites such as phenolic compounds (usually abundant in plants), and for primary metabolites (e.g., sugars and amino acids). This procedure is rapid; it takes not more than 30 min for sample preparation (multiple parallel) and a further 10 min for NMR spectrum acquisition.

Guidelines

TIMING:

In general, sample preparation steps take more time than NMR measurements, and the latter are usually done in an automated system.

Materials

REAGENTS:

- KH_2PO_4 , ACS reagent (Riedel-de Haën, cat. no. 30407)
- 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**269913
- Deuterium oxide (D_2O) > 99.9 atom % D (Spectra Stable Isotopes, cat. no. 5150)
- Methanol-D_4 (D, 99.8%) **Cambridge Isotope Laboratories, Inc. Catalog #**DLM-24-50
- Sodium deuteroxide (D, 99.5%) 40% in D_2O **Cambridge Isotope Laboratories, Inc. Catalog #**DLM-45-50
- Liquid nitrogen

Note

! CAUTION It should be handled carefully and gloves and glasses should be used for protection.

EQUIPMENT:

- Freeze-dryer for sample drying (Edwards Freeze Dryer Modulyo)
- Freezer (−80 °C) for sample storage (RevcoScientific, BV)
- Ultrasonicator (Branson 5510E-MT, Branson Ultrasonics)
- Microcentrifuge (MC-13, Amicon)
- Eppendorf tubes, 2.0 ml (VWR International)
- pH meter (Ankersmit) with electrode (Spintrode, Hamilton)
- 500 MHz Bruker NMR spectrometer (DMX500, Bruker) equipped with a 5 mm TXI probe and a z-gradient system or similar instrument.
- AMIX (Bruker) for bucketing NMR data
- SIMCA-P version 12 (Umetrics AB) or comparable software for multivariate analysis.

REAGENT SETUP:

- **Phosphate buffer** Prepare phosphate buffer (90 mM, pH 6.0) by adding 1.232 g of KH_2PO_4 and 10 mg of TSP (0.01 %) to 100 ml of D_2O. After stirring until total dissolution, adjust the pH using 1.0 M NaOD.
- **1.0 M NaOD** Prepare by adding 1 ml of NaOD (40%, 10 M) to 9 ml of D_2O and mix them well.

EQUIPMENT SETUP:

NMR spectrometer An NMR spectrometer of 500 MHz NMR or higher field strength is suitable for the metabolomic analysis of plants. In general, the MeOD-phosphate buffer extract does not contain those macromolecules that could cause signal broadening in the spectra. Consequently, a ^{1}H NMR measurement protocol with water suppression and a standard pulse sequence is used in metabolomics studies. For the identification of signals, two-dimensional measurements such as J-resolved, ^{1}H–^{1}H COSY and HMBC are necessary. Detailed parameters are described in the PROCEDURE.

Troubleshooting

Problem

The slight shift of certain NMR signals owing to pH or concentration effects can be problematic. Particularly the signals of fumaric acid (singlet, δ 6.50–δ 6.60) and malic acid (δ 2.50–δ 3.00) are well known to vary considerably owing to pH and/or concentration effect. Even in a buffer, which ensures minimum pH shifts, concentration difference in samples can still cause a signal shift. This can be a problem in PCA analysis, where a signal can be recognized as a different metabolite as it bucketed in different bins in different samples. This can be overcome by making a bigger bucket, e.g., 0.1 p.p.m. instead of 0.04 p.p.m. in this area or by removing this area before data analysis (60).

Solution

Not provided

Harvesting of plant

1 Prepare liquid nitrogen in the container (e.g., Dewar barrels) (**Fig. 6**).

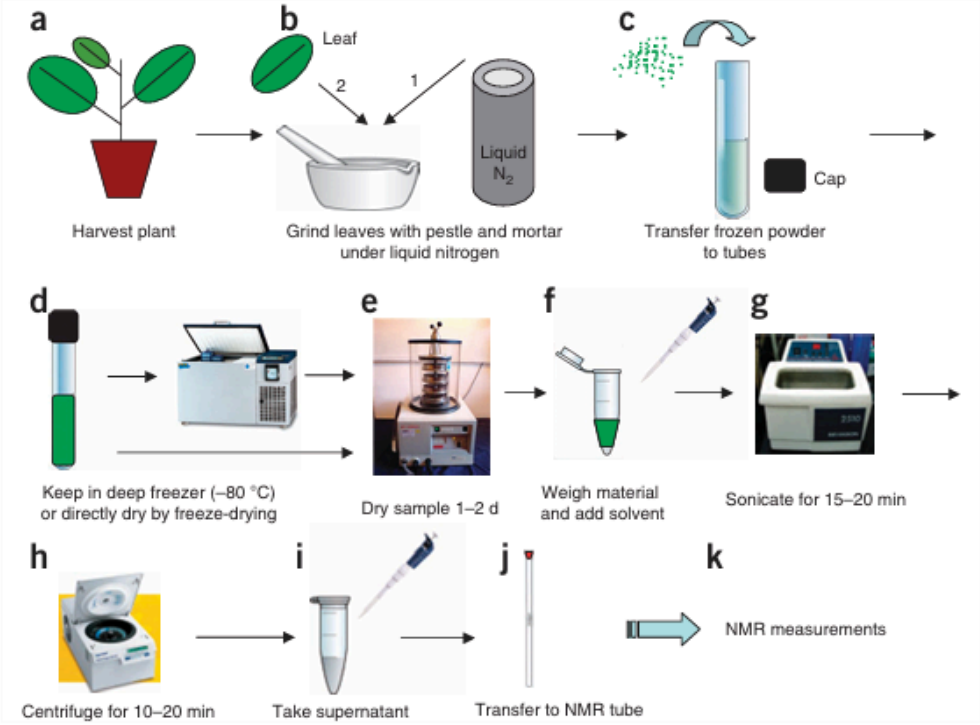

Figure 6: Experimental procedures for sample preparation. (a) Harvest plant. (b) Pre-cool pestle and mortar by adding liquid nitrogen first (1) and place the material in the pestle (2). Grind the materials under liquid nitrogen. (c) Transfer the frozen powder to plastic tubes. (d) Keep frozen samples at − 80 °C or directly dry using freeze-dryer. (e) Dry material for 1–2 d. (f) Weigh dry samples (50 mg) and add extraction solvents (750 µl CD_3OD + 750 µl KH_2PO_4 buffer in D_2O). (g) Extract using ultrasonicator for 10–20 min. (h) Centrifuge at 17,000g at room temperature for 5–10 min to obtain clear supernatant. (i) Collect the supernatant. (j) Transfer 800 µl of supernatant to an NMR tube. (k) Analyze by NMR.

2 Remove the leaves from the plant and, when possible, separate the main veins from the remainder of the leaves (this might not be possible in many plants owing to their small size). Transfer to the liquid nitrogen container in tubes.

Note

PAUSE POINT Frozen tissue can be stored at − 80 °C for several weeks before drying. However, degradation of metabolites might occur during storage. The comparison with fresh material will help to determine whether any degradation takes place or not.

Preparation of freeze-dried samples

3 Grind the frozen leaves using a pre-cooled pestle and mortar under liquid nitrogen (**Fig. 6**).

4 Transfer the ground leaves into a plastic tube using a spatula.

5 Keep it in the deep-freezer before freeze-drying.

6 Place samples in the freeze-dryer for 1–2 d.

Note

! CAUTION Do not leave samples in a freeze-dyer for longer time. Dried sample can absorb moisture easily if new non-dried samples are placed in the dryer.

Note

PAUSE POINT Completely dried samples can be stored at room temperature for several weeks before extraction. A desiccator can be used for the storage of dried samples.

Sample preparation for NMR analysis

31m

7 Weigh the freeze-dried sample in an Eppendorf tube (**Fig. 6**).

8 Add 0.75 mL of CH_3OH-d_4 and 0.75 mL of KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% (wt/wt) TSP.

9 Vortex for 00:01:00 at Room temperature (20 °C – 25 °C). 1m

10 Ultrasonicate for 00:10:00 – 00:20:00 at Room temperature . 20m

11 Centrifuge for 00:05:00 – 00:10:00 using a microtube centrifuge (17000 x g, Room temperature ; *variable speed (14,000–17,000g) can be used to obtain a clear supernatant. For lower-speed centrifugation, more time is required to achieve a clear supernatant). 10m

12 Transfer supernatant (more than 1 mL) to a 1.5 ml Eppendorf tube. If a clear supernatant is not obtained, repeat centrifugation (17000 x g, Room temperature, 00:01:00).

13 Transfer 800 µL of supernatant to a 5mm NMR tube.

Note

PAUSE POINT Extract can be kept for few days in the cold room (0–4 °C) before NMR analysis. However, it is recommended to place at room temperature at least half an hour before NMR measurement to avoid bad shimming owing to the temperature difference in samples.

NMR data acquisition*

14 Load the NMR tube into the spectrometer.

15 Set the sample temperature to 298 °K (25 °C) and leave a few minutes for thermal equilibration.

16 Tune and match the NMR tube.

17 Lock the spectrometer frequency to the deuterium resonance arising from the NMR solvents (either MeOD or D_2O, preferably MeOD).

18 Shim the sample using either manual or automated method.

19 Determine the frequency of the water resonance and set the center of the spectrum to this frequency.

Note

*All these steps are set up in the automation system, but it is recommended to do the first sample manually to obtain good resolved spectra.

20 Select suitable experiments, the options below are for the most frequently used experiments.

20.1 **Standard ^{1}H NMR spectroscopy:**

Set up pulse sequence comprising (relaxation delay-60°-acquire), where pulse power is set to achieve a 60° flip angle, 10 kHz spectral width and water pre-sat applied during 1.5-s relaxation delay. Processing parameters: zero-fill to 64k data points, apply exponential line broadening of 0.3 Hz. After Fourier transformation, manually phase spectrum (zero and first phase), correct baseline and calibrate the spectrum by setting TSP peak at 0.00 p.p.m.

20.2 **J-resolved spectroscopy:**

Setup the J-resolved pulse sequence, two-pulse echo sequence (relaxation delay-90° – [t1/2] – 180° – [t1/2]-acquire) with water pre-sat during a relaxation delay of 1.5 s. Acquire FID using data matrix of 64 × 4,096 points covering 66 × 6,361 Hz, with 16 scans for each increment. Zero-fill the data to 128 × 4,096 and apply a sine bell-shaped window function in both dimensions before magnitude mode two-dimensional Fourier transformation. Tilt the resulting spectra along the rows by 45° relative to the frequency axis and symmetrize about the central line along F2. Manually correct baseline and calibrate to the internal standard (TSP = 0.0 p.p.m.).

20.3 **^{1}H–^{1}H cosY:**

For COSY, use a phase sensitive/magnitude mode standard three pulse sequence with pre-sat during relaxation delay of 1s. A data matrix of 512 × 4,096 points covering 6,361 × 6,361 Hz, record with 8 scans for each increment. Zero fill data to 4,096 × 4,096 points and apply a sine2 bell-shaped window function shifted by /2 in the F1 and /4 in the F2 dimension before States-TPPI type two-dimensional Fourier transformation. Manually phase all spectra, correct baseline and calibrate to the internal standard (TSP = 0.0 p.p.m.).

20.4 **^{1}H–^{13}C HMBC:**

For HMBC spectra, use a data matrix of 254 × 4,096 points covering 27,164 × 6,361 Hz with 256 scans for each increment with a relaxation delay of 1 s. The data should be linear predicted to 512 × 4,096 points using 32 coefficients before magnitude type two-dimensional Fourier transformation and apply a sine bell-shaped window function shifted by /2 in the F1 dimension and /6 in the F2 dimension. Calibrate all spectra according to the internal standard (^{1}H: TSP = 0 p.p.m. and ^{13}C: CD_3OD = 49.0 p.p.m.).

Data analysis

21 Convert NMR spectra to a suitable form for further multivariate analysis. AMIX software is commonly used for converting spectra to an ASCII file. 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 p.p.m. to avoid the effect of signal fluctuation because of pH or concentration. At this stage, signals of remaining solvents have to be removed for the statistical analysis.

22 Carry out PCA using SIMCA-P software (or equivalent softwares, see multivariate data analysis section) as described in the user guides available from Umetrics homepage.

23 Identify as many of the metabolites as possible, either by comparison with NMR signals to reference compounds or by two-dimensional NMR spectra. For more information on the structure elucidation of compounds in mixture, refer to these good reviews[38,39,62].

Sample preparation

1d 12h 30m

24 Harvesting and drying steps depend on the number of samples, as once harvested, homogenization of the sample can be done separately.

1d 12h 30m

- Freeze-drying will take 24:00:00 – 36:00:00, but many samples can be handled simultaneously.
- In the case of extraction, parallel preparation is only limited by the amount and capacity of the equipment involved (i.e., centrifugation); in our case, 24 can be processed at the same time.
- Extraction itself will take 00:20:00 – 00:30:00 including centrifugation and transfer to NMR tubes.

NMR analysis

30m

25 Once the NMR spectrometer is set up for the experiment, ^{1}H-NMR measurement will take 00:05:00 – 00:10:00, depending on the concentration of samples. 30m

- But usually, when the extract is obtained from 50 mg – 100 mg of dry material, 64–128 scans are enough to obtain a good spectrum.
- However, for the first sample, manual shimming and tuning may take up to 00:20:00.
- NMR samples can be loaded in automated systems, which allow, e.g., 24 samples for each run or five times 96 samples if a 'Samplejet' is available (Bruker).

ANTICIPATED RESULTS

26 A typical ^{1}H NMR spectra of a plant extract resulting from the above mentioned extraction protocol is shown in the **Figures 1a–d**. Signals of most primary metabolites are detected in the δ 5.5–δ 0.5 region; amino acids appear around δ 2.0–δ 0.5, organic acids at δ 3.0–δ 2.0 and sugars at δ 5.0–δ 3.0 (**Fig. 1a**; refer also to **Table 2**). The aromatic region comprises many characteristic signals of secondary metabolites. Some examples are as follows:

- Indole compounds such as indole glucosinolate (neoglucobrassicin) and indole acetic acid in Brassica (**Fig. 1b**), indole alkaloids such as catharanthine and vindoline in *Catharanthus roseus* (**Fig. 1c**)
- Phenylpropanoids, flavonoids and aliphatic glucosinolates in *Arabidopsis* (**Fig. 1d**).

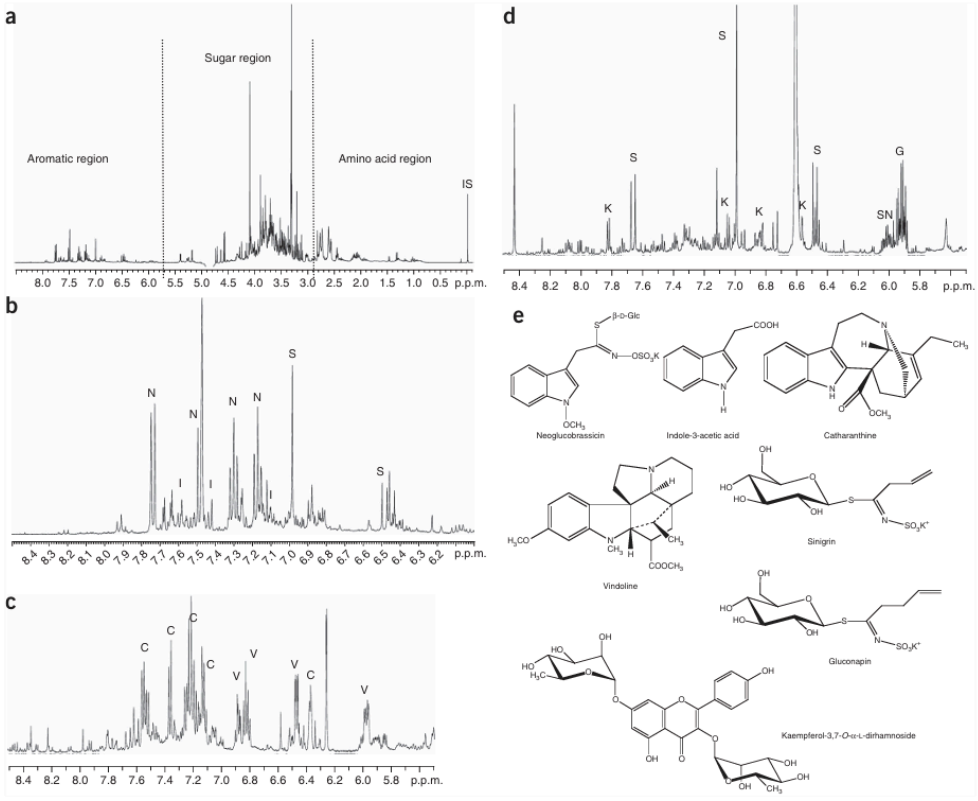

Figure 1: Representative ^{1}H-nuclear magnetic resonance (NMR) spectra of several plant extracts. (a) ^{1}H-NMR spectrum of *Brassica rapa* leaves 14 d after methyl jasmonate treatment. (b) Expended area of a in the range of δ 8.5–δ 6.0. (c) *Catharanthus roseus* leaves in the range of δ 8.5–δ 6.0. (d) *Arabidopsis thaliana* (Columbia) in the range of δ 8.5–δ 6.0. IS: internal standard (3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP)). (e) Chemical structures of metabolites, C: catharanthine, G: gluconapin, I: indole-3- acetic acid, K: kaempferol-3,7-O-α-L-dirhamnopyranoside, N: neoglucobrassicin, S: trans-sinapoylmalate, SN: sinigrin and V: vindoline (adapted Fig. 1a from ref. 95).

Group	Metabolite	Selected characteristic signals in NMR
Sugar	α-Glucose	δ 5.18 (d, *J* = 3.8 Hz)
	β-Glucose	δ 4.58 (d, *J* = 7.8 Hz)
	Sucrose	δ 5.40 (d, *J* = 3.8 Hz), δ 4.17 (d, *J* = 8.5 Hz)
Amino acid	Alanine	δ 1.48 (d, *J* = 7.2 Hz)
	Glutamate	δ 2.07 (m), δ 2.36 (m)
	Glutamine	δ 2.15 (m), δ 2.47 (m)
	Proline	δ 4.08 (dd, *J* = 8.6 Hz, 6.4 Hz), δ 2.34 (m)
	Threonine	δ 1.32 (d, *J* = 6.6 Hz)
	Tryptophan	δ 7.20 (t, *J* = 7.4 Hz), δ 7.29 (t, *J* = 7.5 Hz), δ 7.32 (s), δ 7.54 (d, *J* = 8.1 Hz), δ 7.73 (d, *J* = 7.9 Hz)
	Tyrosine	δ 7.19 (t, *J* = 8.5 Hz), δ 6.86 (t, *J* = 7.5 Hz)
	Valine	δ 1.00 (d, *J* = 6.8 Hz), δ 1.05 (d, *J* = 6.8 Hz)
Organic acid	Citric acid	δ 2.74 (d, *J* = 17.6 Hz), δ 2.56 (d, *J* = 17.6 Hz)
	Formic acid	δ 8.46 (s)
	Fumaric acid	δ 6.56 (s)
	γ-Amino-butyrate (GABA)	δ 1.90 (m), δ 2.30 (t, *J* = 7.2 Hz), δ 3.01 (dd, *J* = 8.4 Hz, 6.3 Hz)
	Malic acid	δ 4.34 (dd, *J* = 6.6 Hz, 4.7 Hz), δ 2.74 (dd, *J* = 16.6 Hz, 4.7 Hz), δ 2.68 (dd, *J* = 16.6 Hz, 6.6 Hz)
	Succinic acid	δ 2.56 (s)
Others	Adenine	δ 8.2 (s), δ 8.21 (s)
	Choline	δ 3.24 (s)
	Inositol	δ 4.00 (t, *J* = 2.8 Hz), δ 3.61 (t, *J* = 9.9 Hz), δ 3.44 (dd, *J* = 9.9 Hz, 2.9 Hz), δ 3.24 (t, *J* = 9.3 Hz)

TABLE 2: Most common metabolites found in plants by nuclear magnetic resonance (NMR) analysis.

27 Different plant extracts showed different profiles. Even for the same plants, considerable metabolite differences can be found depending on their environment and developmental stage. An example is shown in **Figure 2**. Two *Arabidopsis thaliana* of different accessions, grown in the same conditions, showed large difference in their metabolite profiles, not only in their concentrations of individual metabolites but even in the types of metabolites (unpublished data). Congested signals in the ^{1}H-NMR spectra can be simplified using J-resolved spectra as shown in the **Figure 3a**.

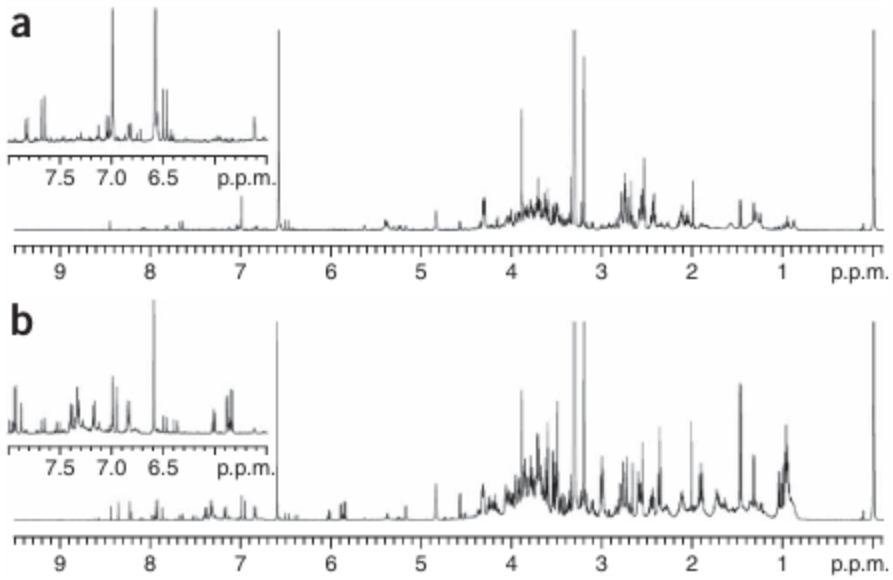

Figure 2: ^{1}H-nuclear magnetic resonance (NMR) spectra of two different accessions of *Arabidopsis* thaliana. Two Arabidopsis were grown in the same condition, harvested at the same developmental stages (4-week-old seedlings) and extracted using the same method. Six plants were combined when harvested. (a) *Arabidopsis thaliana* Col-0. (b) *Arabidopsis thaliana* C24 (unpublished data).

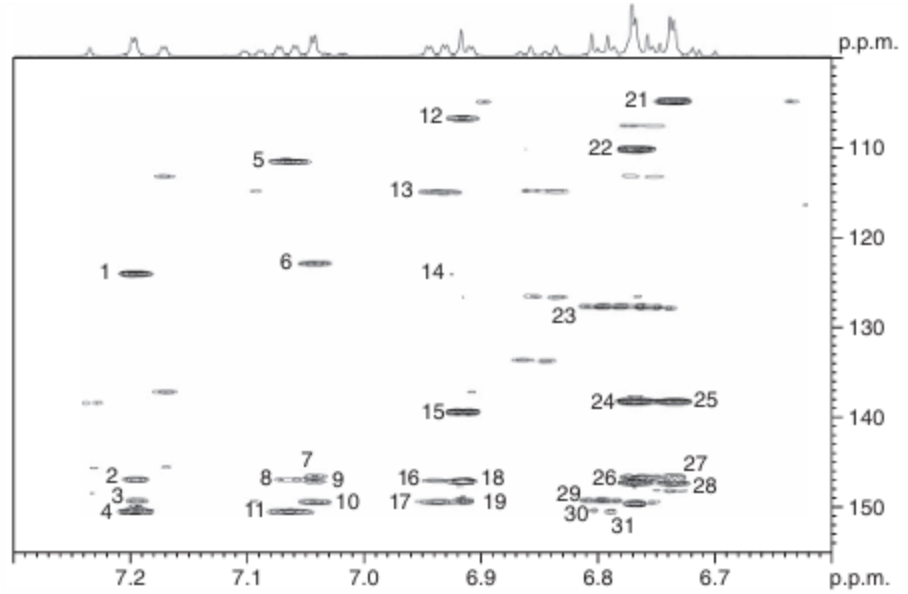

Figure 3: J-resolved nuclear magnetic resonance (NMR) spectra of methyl jasmonate (MJ)-treated Brassica rapa leaves. (a,b) In the region of δ 8.5–δ 6.0 (a) and their chemical structures (b) 1: neoglucobrassicin, 2: hydroxycinnamates malate, 3: indole-3-acetic acid, 4: trans-sinapoylmalate, 5: cis-sinapoylmalate, 6: cis-feruloylmalate, 7: cis-coumaroylmalate, 8: trans-feruloylmalate and 9: trans-coumaroylmalate (adapted from ref. 95).

28 By making a projection on the spectral axis of these two-dimensional spectra, multiplet signals become singlets, giving much clearer and sharper signals and thus considerably facilitating peak identification (compare **Fig. 1b and Fig. 3a**).

29 In one of our studies on *Brassica*, a metabolomic approach was carried out to obtain a holistic picture of metabolic changes in *Brassica* after methyl jasmonate (MJ) treatment[95]. Samples were collected at different time points after MJ treatment and their NMR signals were compared with corresponding control samples. PCA results showed that MJ treatment produced metabolite changes after 2 d and further changes continued for the 14 d of experiment. Control groups showed different metabolite profiles to MJ-treated groups (**Fig. 7**).

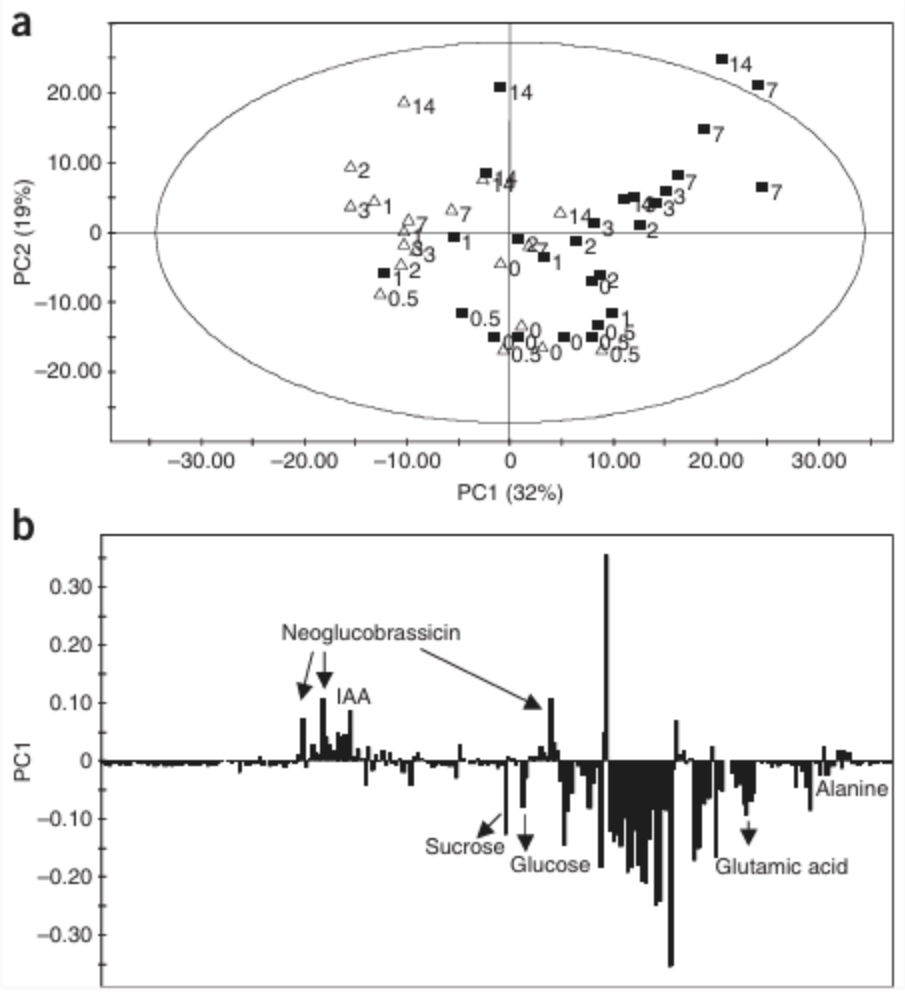

Figure 7: Metabolites changes of *Brassica rapa* leaves after methyl jasmonate (MJ). (a,b) Score plot of principal component analysis (PCA) of (a) J-resolved nuclear magnetic resonance (NMR) data of *Brassica rapa* and (b) loading plot of PC1. Control plants are shown as open triangles (Δ) and MJ-treated plants are shown as solid box (□). The number after the symbol shows the time (d) after MJ treatments (adapted from ref. 95).

30 In MJ-treated *Brassica*, several phenolic compounds were observed to increase if compared with control groups. Considerable signal overlapping hampered the identification of individual compounds in the crude extract. To isolate increased metabolites, the crude extract was submitted to further column chromatography. The first separation on HP20 yielded five fractions, the last of which containing phenolic compounds (**Fig. 8a**) was further subjected to Sephadex LH-20 column chromatography. In the isolated fraction, ten different malate-conjugated phenylpropanoids were detected[96] (**Fig. 8b**). Their structures were identified using their two-dimensional NMR spectra, and the HMBC spectrum is shown in **Figure 4**.

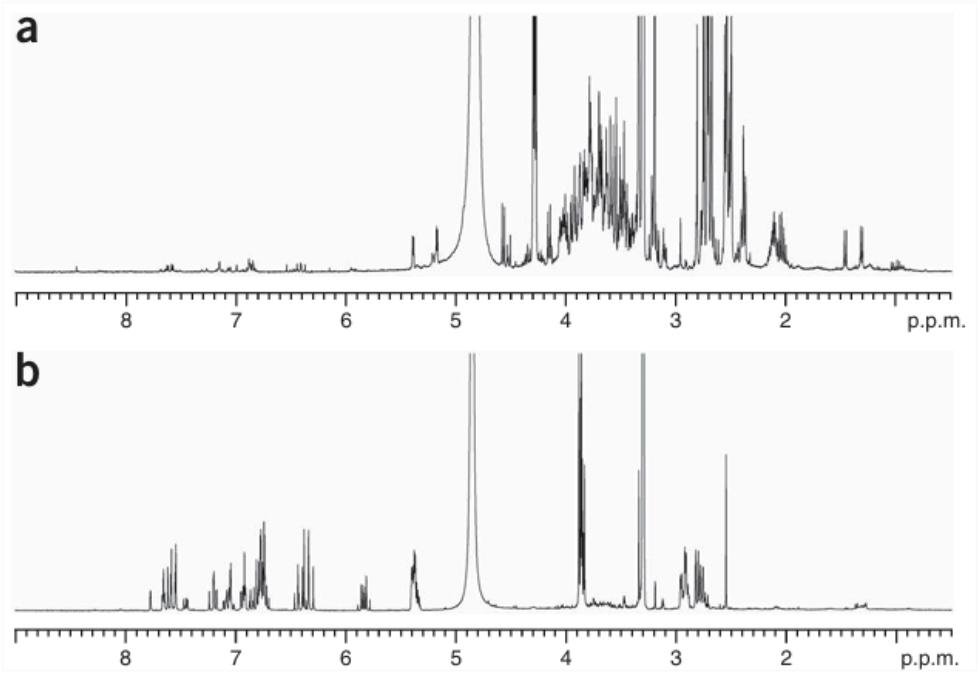

Figure 8: ^{1}H-nuclear magnetic resonance (NMR) spectra of methyl jasmonate (MJ)-treated *Brassica rapa* leaves (a,b). (a) The fifth fraction containing phenylpropanoids before Sephadex LH-20 column chromatography and (b) isolated fraction by eluting with methanol. The heteronuclear multiple bond correlation (HMBC) spectrum of B is shown in Figure 4. The sugars and other metabolites are considerably removed by Sephadex LH-20 column.

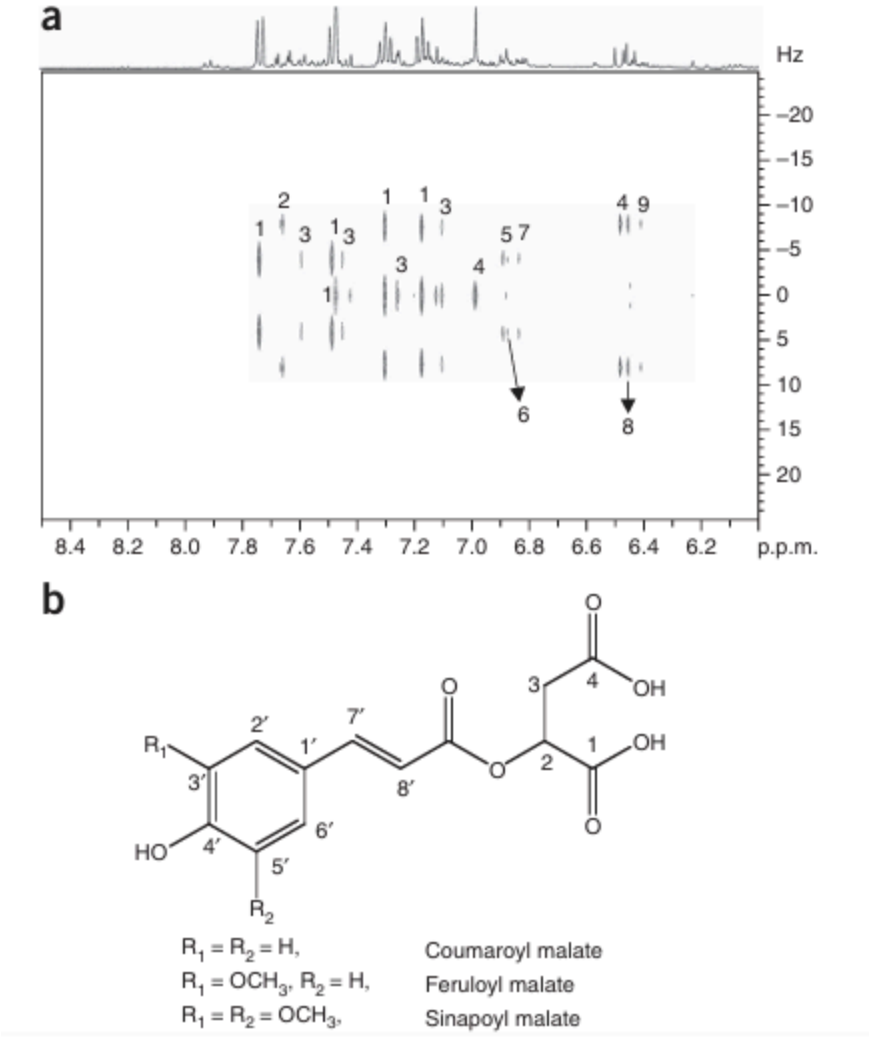

Figure 4: Heteronuclear multiple bond correlation (HMBC) spectrum of aromatic moiety of phenylpropanoids of *Brassica rapa* leaves in the range of δ 6.60–δ 7.30 of 1H and δ 100–δ 150 of ^{13}C. 1: H-2'/C-6' of feruloyl malate, 2: H-2'/C-7' of feruloyl malate, 3: H-2'/C-3' of feruloyl malate, 4: H-2'/C-4' of feruloyl malate, 5: H-6'/C-2' of feruloyl malate, 6: H-2'/C-6' of caffeoyl malate, 7: H-2'/C-3' of caffeoyl malate, 8: H-6'/C-7' of feruloyl malate, 9: H-2'/C-7' of caffeoyl malate, 10: H-2'/C-4' of caffeoyl malate, 11: H-6'/C-4' of feruloyl malate, 12: H-2' and 6'/C-2' and 6' of sinapoyl malate, 13: H-6'/C-2' of caffeoyl malate, 14: H-2' and 6'/C-1' of sinapoyl malate, 15: H-2' and 6'/C-4' of sinapoyl malate, 16: H-6'/C7' of caffeoyl malate, 17: H-6'/C-4' of caffeoyl malate, 18: H-2' and 6'/C-7' of sinapoyl malate, 19: H-2' and 6'/C-3' and 5' of sinapoyl malate, 20: H-6'/C-2' of 5-hydroxyferuloyl malate, 21: H-2'/C-6' of 5-hydroxyferuloyl malate, 22: H-5'/C-1' of feruloyl malate, 23: H-2'/C-4' of 5-hydroxyferuloyl malate, 24: H-6'/C-4' of 5-hydroxyferuloyl malate, 25: H-2'/C-7' of 5-hydroxyferuloyl malate, 26: H-6'/C 5' of 5-hydroxyferuloyl malate, 27: H-6'/C-7' of 5-hydroxyferuloyl malate, 28: H-5'/C-3' of feruloyl malate, 29: H-5'/C-4' of feruloyl malate and 30: H-2'/C-3' of 5-hydroxyferuloyl malate (adapted from ref. 96).

31 Another interesting example was the determination of metabolites at different time points in tobacco leaves (local leaves) after infection with TMV and systemic leaves. Leaves

above the infected leaves showed an increased resistance against TMV virus after the infection (systemic acquired resistance (SAR)). Moreover, different metabolic changes were observed in local and systemic leaves after leaf infection (**Fig. 9**)[29]. Thus, by measuring many metabolites simultaneously in one analysis, both quantitatively and qualitatively, the metabolomic approach provides a snapshot of the plant metabolism after infection. The metabolic changes are summarized in the biosynthetic pathways as shown in **Figure 10**.

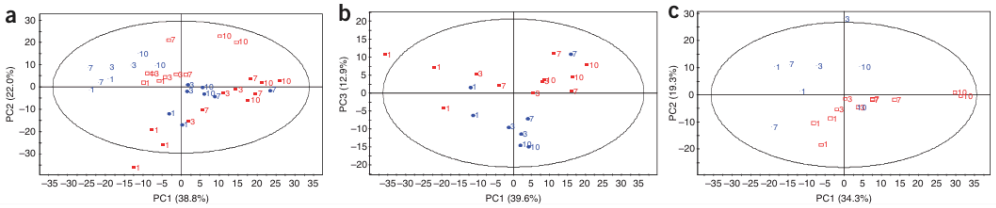

Figure 9: Metabolites changes of *Nicotiana tabacum* leaves after tobacco mosaic virus (TMV) infection. (a,b) Score plot of principal component analysis of (a) healthy (lower and upper) and (b) infected (local-infected and systemic acquired resistant) *Nicotiana tabacum* leaves by tobacco mosaic virus, and (c) loading plot (PC2) of infected leaves. , Lower leaves of healthy plants; , upper leaves of healthy plants; , local-infected leaves of TMV-infected plants;, systemic-acquired resistance leaves of TMV-infected plants. (a) Principal component analysis (PCA) for lower and upper leaves in healthy plants and inoculated and systemic acquired resistant leaves, (b) PCA for lower leaves in healthy plants and inoculated leaves in the infected plants, (c) PCA for upper leaves in healthy plants and systemic-acquired resistant leaves in the infected plants. Numbering in plot A and B are the dates after infection. The number of 1 and 4 on plot C are nicotine and 5-caffeoyl quinic acid signals, respectively (adapted from ref. 29).

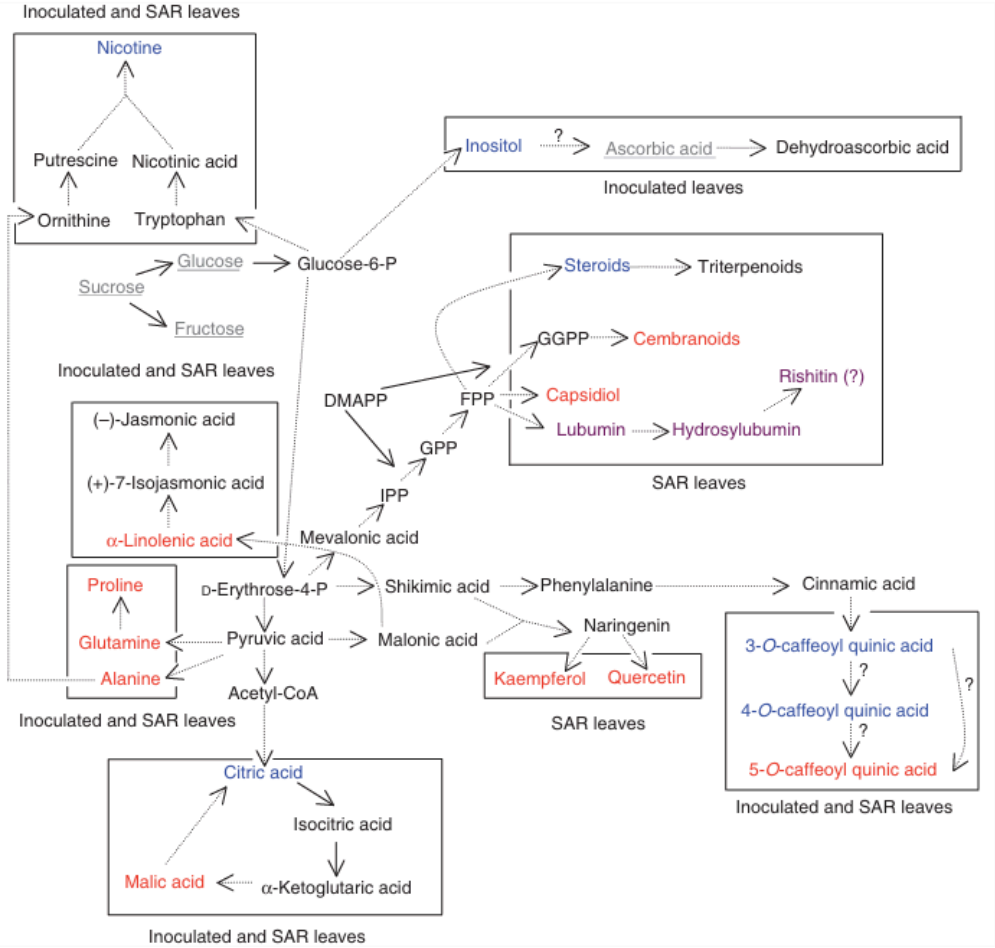

Figure 10: Proposed metabolomic alterations in the *Nicotiana tabacum* leaves infected by tobacco mosaic virus. Blue (•): decreased; red (•): increased; gray (•): transient increased; purple (•): previous results from other *Nicotiana* species (e.g. *Nicotiana undulata* or *Nicotiana rustica* and *Nicotiana glutinosa*); and black (•): based on general plant biosynthesis (adapted from ref. 29).

Protocol references

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