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Metabolomic analysis of methyl jasmonate treated *Brassica rapa* leaves by 2-dimensional NMR spectroscopy

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Abstract

The metabolomic analysis of *Brassica rapa* leaves treated with methyl jasmonate was performed using 2-dimensional *J*-resolved NMR spectroscopy combined with multivariate data analysis. The principal component analysis of the *J*-resolved NMR spectra showed discrimination between control and methyl jasmonate treated plants by principal components 1 and 2. While the level of glucose, sucrose and amino acids showed a decrease after methyl jasmonate treatment, hydroxycinnamates and glucosinolate were highly increased. Methyl jasmonate treatment resulted in a long-term accumulation of indole glucosinolate and indole-3-acetic acid, lasting up to 14 days after treatment. Malate conjugated hydroxycinnamates also exhibited an increase until 14 days after methyl jasmonate treatment, these compounds might play an important role in plant defence responses mediated by methyl jasmonate.

Keywords: *Brassica rapa*; Brassicaceae; Metabolomic analysis; *J*-resolved NMR; Multivariate analysis; Indole glucosinolate; Hydroxycinnamates

1. Introduction

Jasmonic acid (JA) and its methyl ester (MJ) have been shown to have a variety of physiological functions in plants including an involvement in developmental processes and defence responses to insects, pathogens, wounding and desiccation (Creelman and Mullet, 1997; Weber, 2002; Farmer et al., 2003).

It is known that herbivore attack and injury cause an increase of endogenous JA, which plays a role as a regulatory signal to produce defensive proteins (e.g. PR protein) and metabolites (e.g. phytoalexins) (Farmer et al., 2003; Zhao et al., 2005). Exogenous application of JA to a plant cell culture or intact plant stimulates the biosynthesis of a wide variety of plant secondary metabolites, each plant species seems to induce different specious specific type of secondary metabolites pathway (Gundlach et al., 1992; Zhao et al., 2005). For this reason, JA treatment is considered to be a very useful biochemical tool to elicit the production of secondary metabolites and to study plant defence responses.

Brassica genus includes species of great economical importance since they are used as vegetables or as a source of edible oil (Bennett and Wallsgrove, 1994). The investigation of various aspects of plant insect interactions and plant defence mechanisms has therefore awakened great interest (Pedra, 1998). Up to now most of these chemical and molecular/biological studies are focused on glucosinolates which are found exclusively in this family and the effect of JA on their production constitutes one of the major research interests. For example, in white mustard (*Sinapis alba*), MJ has been shown to increase the

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incorporation rate of ^{14}C-Tyr into *p*-hydroxybenzylglucosinolate (Du et al., 1995). Oilseed rape (*Brassica napus*) treated either with MJ or by injury has been shown to accumulate increased levels of indol-3-yl-methyl glucosinolate (Bodnaryk, 1994). However, holistic approaches to detect a wide range of metabolite changes due to the effect of MJ have not yet been performed.

Metabolomics, which is defined as a comprehensive quantitative and qualitative analysis of all metabolites within cells, tissues or organs, can be instrumental in providing the information necessary for this type of approach. However, due to the complexity and diversity of plant metabolites it is unlikely that one single analytical method could generate information about all metabolites present in a plant and it could probably be necessary to perform a wide range of chemical analysis which should be both rapid and reproducible. High-resolution ^{1}H NMR is one of such methods, constituting a promising tool with a potential to detect and identify a large number of compounds it is, thus, a leading technique in the emerging area of metabolomic studies. So far, ^{1}H NMR has been successfully used in the area of toxicology and clinical diagnostics (Nicholson et al., 2002; Brindle et al., 2002; Keun, 2006). Several studies were reported in the field of plant metabolites, applying them to the discrimination of different species/ecotypes of plants (Ward et al., 2003; Kim et al., 2005), in the differentiation of transgenic plants and their wild type counterpart (Choi et al., 2004a) or to distinguish infected plants from healthy plants (Choi et al., 2004b).

Although ^{1}H NMR is a very powerful tool in metabolic analysis, it has several disadvantages due to its relatively low resolution and the congestion of signals in certain parts of the spectra when applied to the analyses of mixtures. Thus, when working with complex mixtures it is crucial to reduce spectral overlapping. 2D NMR is an attractive solution for this problem. Among 2D NMR techniques, *J*-resolved ^{1}H NMR is quite promising since it greatly improves the resolution of the ^{1}H NMR spectra in a relatively short time (25 min) compared to the other available 2D NMR techniques. *J*-resolved spectra separate the chemical shift and spin–spin coupling data onto different axes, F1 for spin–spin coupling and F2 for chemical shifts. Thus it provides spin multiplicities which are sometimes difficult to determine in the 1D ^{1}H NMR due to overlapping of signals. It also is easy to build up a database since a projection of the 2D spectrum on the chemical shift axis results in a spectrum in which the protons are observed as singlet (Viant, 2003).

The purpose of this study was to examine metabolic changes of *Brassica rapa* leaves after MJ treatment using 2D NMR spectroscopy (especially *J*-resolved ^{1}H NMR) combined with multivariate data analysis. This method allows the detection of a large number of metabolites which are affected by MJ. The results suggest that several types of metabolites such as hydroxycinnamates, indole derivatives and glucosinolates may play an important role in plant defence mechanisms.

2. Results and discussion

2.1. Identification of metabolites in MJ treated Brassica rapa leaves

CD_3OD–D_2O extracts of *Brassica rapa* var. *rapa* leaves were analyzed by ^{1}H NMR. Fig. 1 shows the spectra of *Brassica* plant extracts of control and MJ treated leaves (14 days after MJ treatment). There are very notable differences in the NMR of the MJ treated plants, especially in the aromatic region. The sample was exhaustively analyzed using 2D experiments such as ^{1}H–^{1}H COSY, *J*-resolved ^{1}H NMR, HMQC and HMBC. The combined spectral information thus obtained from the 2D experiments and an in-house library of ^{1}H NMR spectra of reference compounds allowed an almost complete assignment of the signals. The chemical shifts and structures of the metabolites of *Brassica* leaves detected by ^{1}H NMR are listed in Table 1 and Fig. 2. 2D *J*-resolved spectroscopy was very useful to interpret signals from congested regions like the aromatic region of the MJ treated plant extract, because it shows coupling constants on the other axis, thus allowing the detection of splitting patterns and coupling constants of each signal (Fig. 3).

In the aromatic region (Fig. 3a), 2D *J*-resolved NMR clearly shows the presence of two indole compounds (neoglucobrassicin and indole 3-acetic acid), which were found only in MJ treated plants. *Trans*/*cis*-hydroxycinnamates (sinapoylmalate, feruloylmalate and coumaroylmalate)

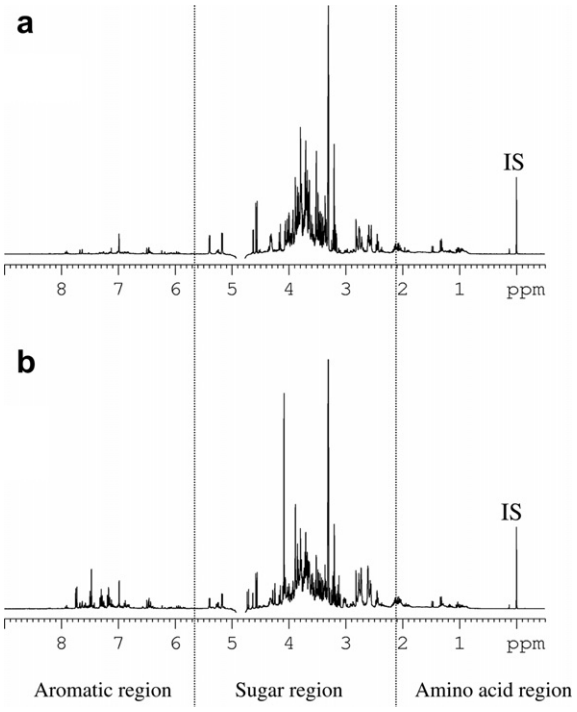


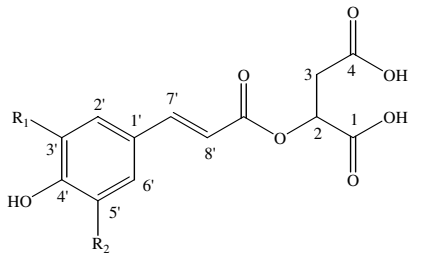
Fig. 1. ^{1}H NMR spectra of methanol/water extraction of control (a) and MJ treated (b) *Brassica rapa* leaves (14 days after treatment). IS, internal standard (TSP).

Table 1
Selected characteristic ^{1}H Chemical shifts of metabolites of MJ treated *Brassica rapa* leaves detected from 1D and 2D spectra

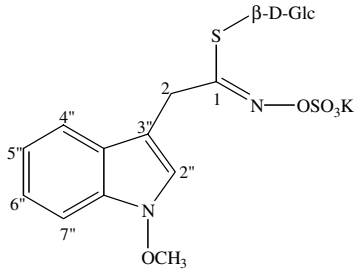
Metabolite	Chemical shift (δ) and coupling constant (Hz)
trans-Sinapoylmalate	7.63 (*d*, $J = 16.0$, H-7′), 6.98 (*s*, H-2′, H-6′), 6.49 (*d*, $J = 16.0$, H-8′), 5.26 (*dd*, $J = 9.2$, 3.6, H-2), 2.90 (*dd*, $J = 16.0$, 3.6, H-3α), 2.84 (*dd*, $J = 16.0$, 3.6, H-3β)
trans-Feruloylmalate	7.63 (*d*, $J = 16.0$, H-7′), 6.46 (*d*, $J = 16.0$, H-8′), 5.26 (*dd*, $J = 9.2$, 3.6, H-2), 2.90 (H-3a, dd, $J = 16.0$, 3.6), 2.84 (H-3β, dd, $J = 16.0$, 3.6)
trans-Coumaroylmalate	7.63 (*d*, $J = 16.0$, H-7′), 6.41 (*d*, $J = 16.0$, H-8′), 5.26 (*dd*, $J = 9.2$, 3.6, H-2), 2.90 (*dd*, $J = 16.0$, 3.6, H-3a), 2.84 (*dd*, $J = 16.0$, 3.6, H-3β)
cis-Sinapoylmalate	6.89 (*d*, $J = 12.0$ H-7′), 5.93 (*d*, $J = 12.0$, H-8′), 5.23 (*dd*, $J = 9.2$, 3.6, H-2)
cis-Feruloylmalate	6.88 (*d*, $J = 12.0$, H-7′), 5.95 (*d*, $J = 12.0$, H-8′), 5.23 (*dd*, $J = 9.2$, 3.6, H-2,)
cis-Coumaroylmalate	6.84 (*d*, $J = 12.0$, H-7′), 5.97 (*d*, $J = 12.0$, H-8′), 5.23 (*dd*, $J = 9.2$, 3.6, H-2)
Neoglucobrassicin	7.72 (*d*, $J = 7.8$, H-4″), 7.47 (*d*, $J = 7.8$, H-7″), 7.46 (*s*, H-2″), 7.29 (*t*, $J = 7.8$, H-6″), 7.16 (*t*, $J = 7.8$, H-5″), 4.72 (*d*, $J = 9.8$, H-1′), 4.25 (*d*, $J = 16.0$, H-2a), 4.09 (*d*, $J = 16.0$, H-2b), 4.07 (*s*, $-OCH_3$,)
Indole 3-acetic acid	7.59 (*d*, $J = 7.8$, H-4), 7.45 (*d*, $J = 7.8$, H-7), 7.27 (*t*, $J = 7.8$, H-6), 7.12 (*s*, H-2), 7.10 (*t*, $J = 7.8$, H-5), 3.38 (*d*, $J = 16.0$, H-2α), 3.23(*d*, $J = 16.0$, H-2β)
Sucrose	5.40 (*d*, $J = 3.9$, H-1)
α-Glucose	5.18 (*d*, $J = 3.8$, H-1)
β-Glucose	4.58 (*d*, $J = 7.8$, H-1)
Malic acid	4.34 (*dd*, $J = 6.6$, 4.7, H-2), 2.74 (*dd*, $J = 16.6$, 4.7, H-3α), 2.68 (*dd*, $J = 16.6$, 6.6, H-3β)
Succinic acid	2.56 (*s*)
Glutamic acid	3.73 (*t*, $J = 7.0$, H-2), 2.45 (*td*, $J = 7.6$, 2.1, H-4), 2.14 (*m*, H-3α), 2.06 (*m*, H-3β)
Alanine	1.48 (*d*, $J = 7.2$, H-3)
Threonine	1.34 (*d*, $J = 6.6$, H-4)
1-*O*-ethyl-β-glucoside	1.19 (*t*, $J = 7.0$, H-2′)
Valine	1.00 (*d*, $J = 6.8$, H-4α), 1.05 (*d*, $J = 6.8$, H-4β)

were found in both extracts, but in a higher levels in MJ treated plants. *Cis*-hydroxycinnamates are considered to be artifacts produced by isomerisation of their *trans* isomers (Liang et al., 2006).

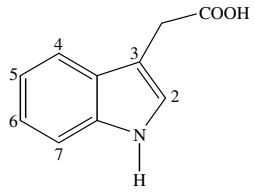
In the sugar region (Fig. 3b), signals from glycosidically bound glucose were found apart from ubiquitious carbohydrates such as α-, β-glucose and sucrose. The anomeric signal of glucose in glucosinolates is very noticeable. Based on its chemical shift, the type of glucosinolates present (aliphatic, aromatic and indole glucosinolates) could be deduced. The chemical shifts for the anomeric glucose protons in aliphatic glucosinolates appear at δ 5.1–5.0, independently of the structure of the side chain. On the other hand, anomeric glucose protons of aromatic or indole glucosinolates are more upfield (δ 4.7–5.0), presumably due to aromatic ring current effects (Prestera et al., 1996). Another characteristic of glucosinolates is that the anomeric proton of glucose (*S*-Glc) shows a larger coupling constant (9.5–10 Hz) than that of the β-glucose ($J = 7$–8 Hz) in *O*-Glc. Additionally, this proton shows correlation with C-1 at δ 160–165 in the HMBC spectrum. These characteristics can, therefore, be applied to identify glucosinolates in the spectra of the mixture.



$R_1 = R_2 = H$, Coumaroyl malate
$R_1 = OCH_3$, $R_2 = H$, Feruloyl malate
$R_1 = R_2 = OCH_3$, Sinapoyl malate



Neoglucobrassicin



Indole-3-acetic acid

Fig. 2. The chemical structure of hydroxycinnamoyl malates and induced indole compounds found in the MJ treated *Brassica rapa* leaves.

In the organic acid region (Fig. 3c), malic acid, succinic acid and glutamic acid were detected. Malic acid conjugated with *trans*/*cis*-hydroxycinnamates were also found in this region.

In the amino acid region (Fig. 3c), three amino acids, alanine, threonine and valine were found in both control and MJ treated plants. In addition, the signal from 1-*O*-ethyl-β-glucoside was found at δ 1.19 (*t*, $J = 7.0$ Hz) in both extracts, presumably due to glucosylation of the EtOH used as solvent vehicle for MJ (Kraemer et al., 1999).

2.2. Metabolite profile changes after methyl jasmonate treatment – PCA analysis

After qualitative analysis of the NMR spectra, further analyses using principal component analysis (PCA) to

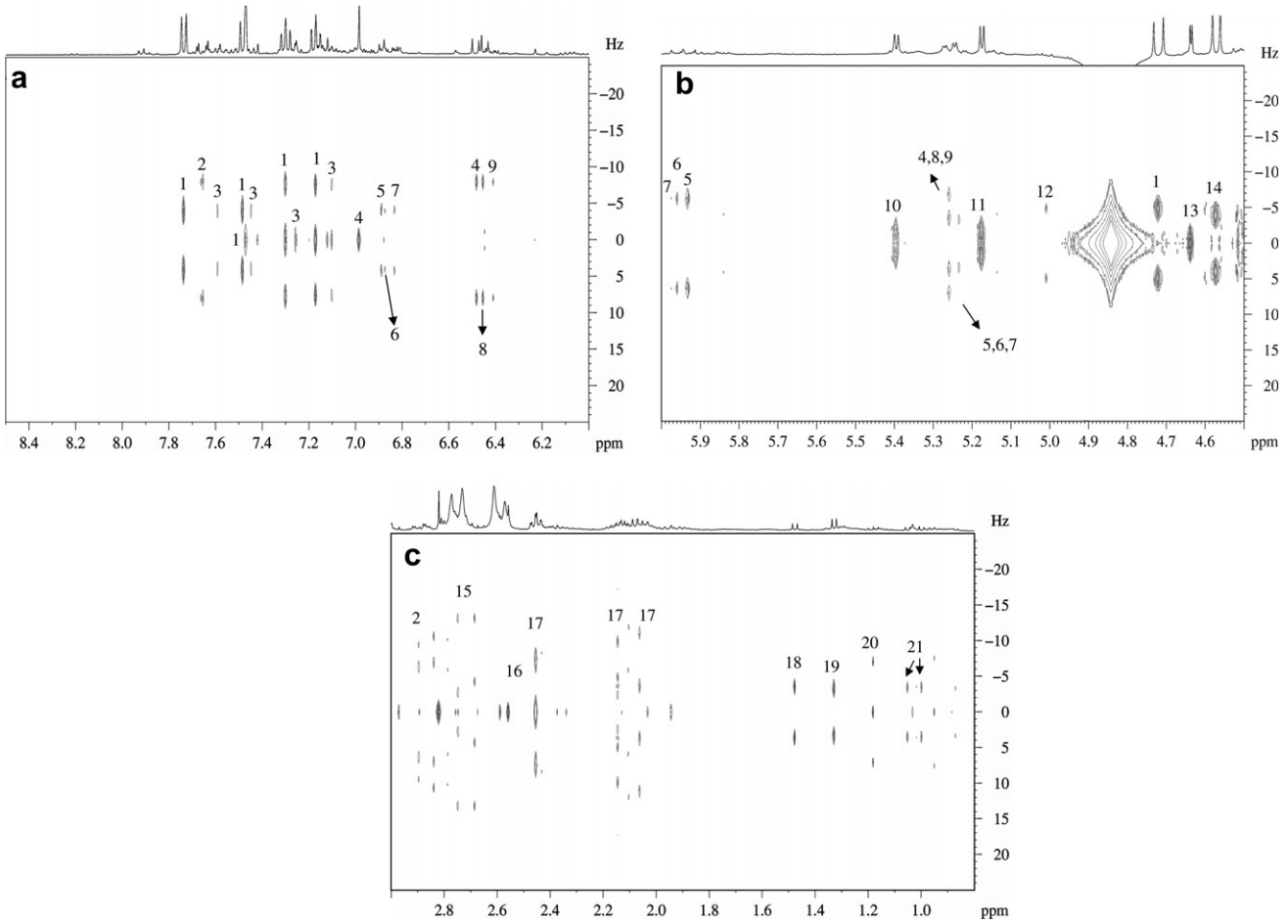


Fig. 3. *J*-resolved NMR spectra of MJ treated *Brassica rapa* leaves. In the region of δ 8.5–6.0 (a), δ 6.0–4.0 (b) and δ 3.0–0.8 (c). Peak assignments: (1) neoglucobrassicin, (2) hydroxycinnamates malate, (3) indole-3-acetic acid, (4) *trans*-sinapoylmalate, (5) *cis*-sinapoylmalate, (6) *cis*-feruloylmalate, (7) *cis*-coumaroylmalate, (8) *trans*-feruloylmalate, (9) *trans*-coumaroylmalate, (10) sucrose, (11) α-glucose, (12) aliphatic glucosinolate, (13) apiose of phenolic glycoside, (14) β-glucose, (15) malic acid, (16) succinic acid, (17) glutamic acid, (18) alanine, (19) threonine, (20) 1-*O*-ethyl-β-glucoside, (21) valine. Assignments of each signal were listed in Table 1.

monitor metabolite changes in the leaves of MJ treated plants were performed. We used the dataset of projected *J*-resolved spectra for PCA, in which all of signals appear as singlet. The scatter plot showed discrimination between two groups by principal component (PC) 1 and 2 (Fig. 4a). MJ treated plants showed a positive PC1 value, while most of control plants showed a negative PC1 value. Compared to the earlier time point (less than 1 day after treatment), plant extracts obtained at a later stage are clearly discriminated from control plants by PC1. The loading plot of PC1 (Fig. 4b) explains that the signals from neoglucobrassicin (δ 7.72, 7.48, 7.16, 4.72, 4.08), IAA (δ 7.60) and hydroxycinnamate derivatives (δ 7.64, 6.40, 6.92) have a positive effect in PC1. In contrast, the signals from sucrose (δ 5.40), β-glucose (δ 5.20), α-glucose (δ 4.56), free malic acid (δ 2.74, 2.68) and glutamic acid (δ 2.48, 2.14, 2.06) show negative PC1 values. It means that after MJ treatment, neoglucobrassicin, IAA and hydroxycinnamate derivatives are increased compared to the control group, whereas sucrose, glucose and amino acids such as alanine, threonine and glutamic acid are decreased.

The changes in some primary metabolites which contributed to PC2, such as glutamic acid (negative PC2), sucrose and glucose (both positive PC2) seems also related to developmental stages since both control and MJ treated plants showed increased PC2 value in time (Fig. 4a).

To examine the MJ effect in detail, PCA was performed using the *J*-resolved ^{1}H NMR data of MJ treated plants only (Fig. 5a). It can be clearly seen that there was a metabolomic change in the MJ treated plants after one day. After one and two days of MJ treatment, plants showed higher PC2 values and later on, 3, 7 and 14 days after treatment, plants showed higher PC1 values. The loading plots of PC1 and PC2 (Fig. 5b) indicated that a higher amount of malic acid, alanine, threonine, glutamic acid and sucrose was present in the early stage and neoglucobrassicin, IAA and hydroxycinnamates levels were higher in the later stages (day 3, 7 and 14).

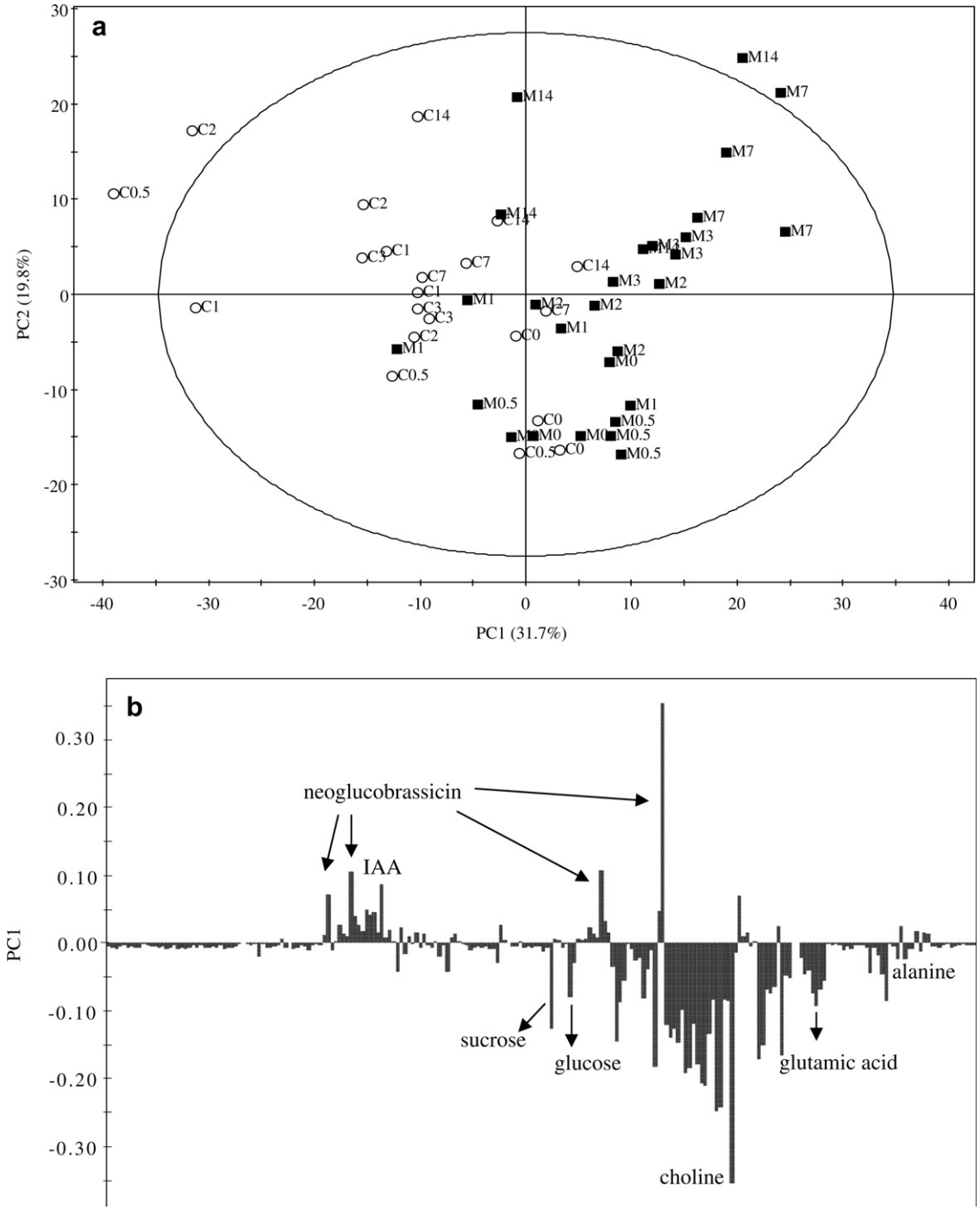


Fig. 4. Score plot of PCA of *J*-resolved NMR data of *Brassica rapa* (a) and loading plot of PC1 (b). Control plants are shown as open circle (○) and MJ treated plants are shown as solid box (■). The number after symbol shows the time (day) after MJ treatments.

2.3. Metabolite profile changes after methyl jasmonate treatment – quantitative analysis

To determine the amount of each compound which contributed to the discrimination between control and MJ treated plants, each signal was integrated (See Fig. 6). Results showed that sugar and amino acids content was clearly decreased shortly after MJ treatment, whereas neoglucobrassicin, IAA and hydroxycinnamates were increased. Neoglucobrassicin was found at a higher level 7 days after MJ treatments and remained at those high levels throughout the 14 days. Similar results were reported for oilseed rape (Bodnaryk, 1992; Doughty et al., 1995) and *Arabidopsis* (Brader et al., 2001), where neoglucobrassicin was found to be increased after treatment with MJ or wounding. Rostás et al. (2002) also reported that herbivore and fungal infection exclusively increased the level of neoglucobrassicin in Chinese cabbage. These results clearly

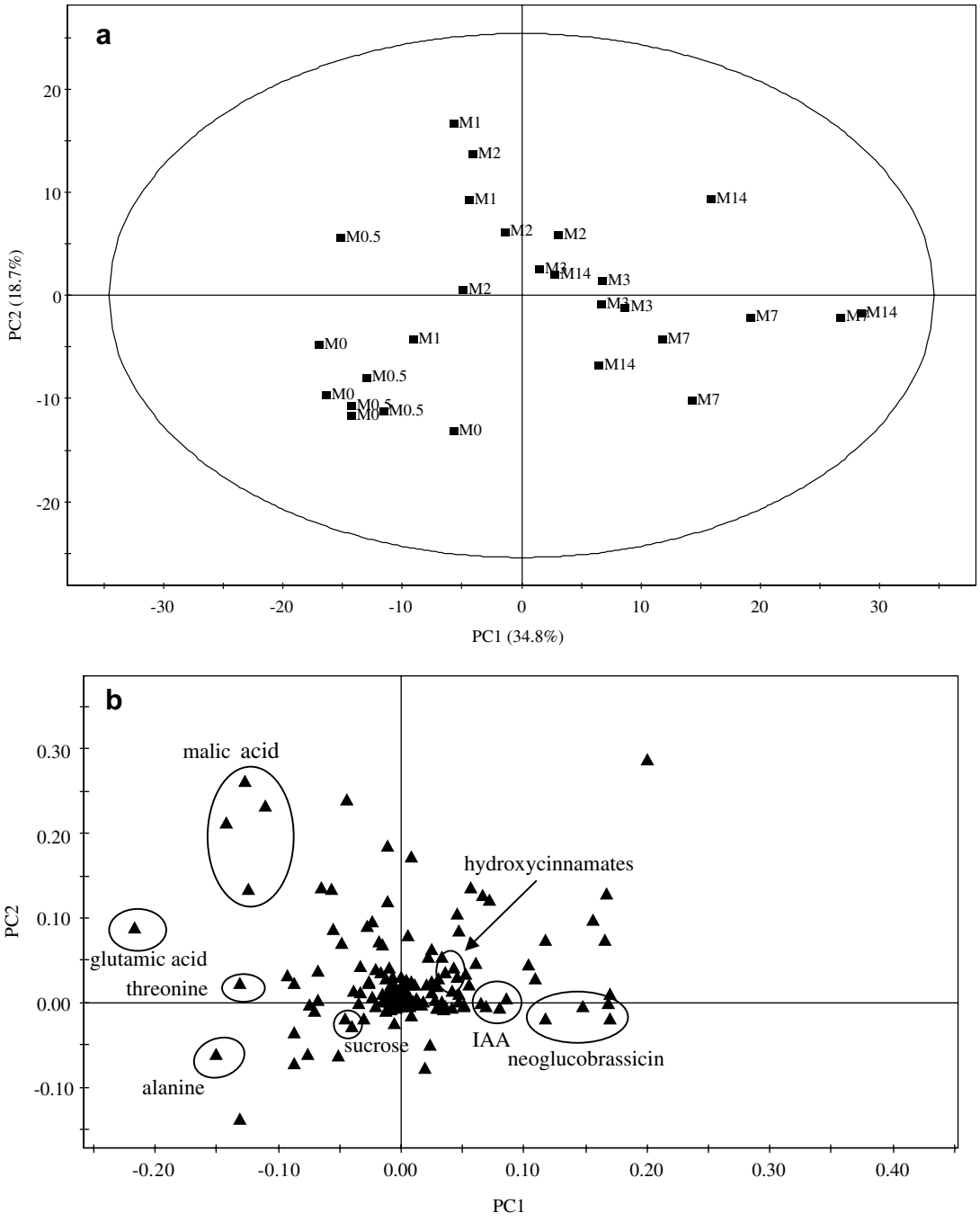


Fig. 5. Score plot of PCA of *J*-resolved NMR data of *Brassica rapa* of MJ treated plants (a) and loading plot of PC1 and PC2 (b). The number after symbol (■) indicates the time (day) after MJ treatments.

show the involvement of neoglucobrassicin in the plant defence response.

Besides neoglucobrassicin, we found that IAA also increased after MJ treatment. IAA is an important plant hormone controlling a variety of developmental processes (Weiler et al., 1993). Furthermore, it has been reported that IAA has an antimicrobial activity against several species of bacteria (Lu et al., 2002). In most higher plants, the biosynthesis of IAA proceeds via two tryptophan dependent pathways, i.e. the indole-3-acetonitrile pathway and the indole pyruvic acid pathway (Chen and Andreasson, 2001). In addition, for Brassicaceae, it has been reported that indole glucosinolates can be converted by myrosinases into indole-3-acetonitrile and further hydrolyzed to IAA by nitrilases (Bartel and Fink, 1994). MJ treatment may induce the tryptophan pathway in *Brassica*, leading to the production of IAA and neoglucobrassicin as we found in our studies. There is still the possibility that IAA is produced from indole glucosinolate, e.g., glucobrassicin by myrosinase (Bartel and Fink, 1994). However, considering

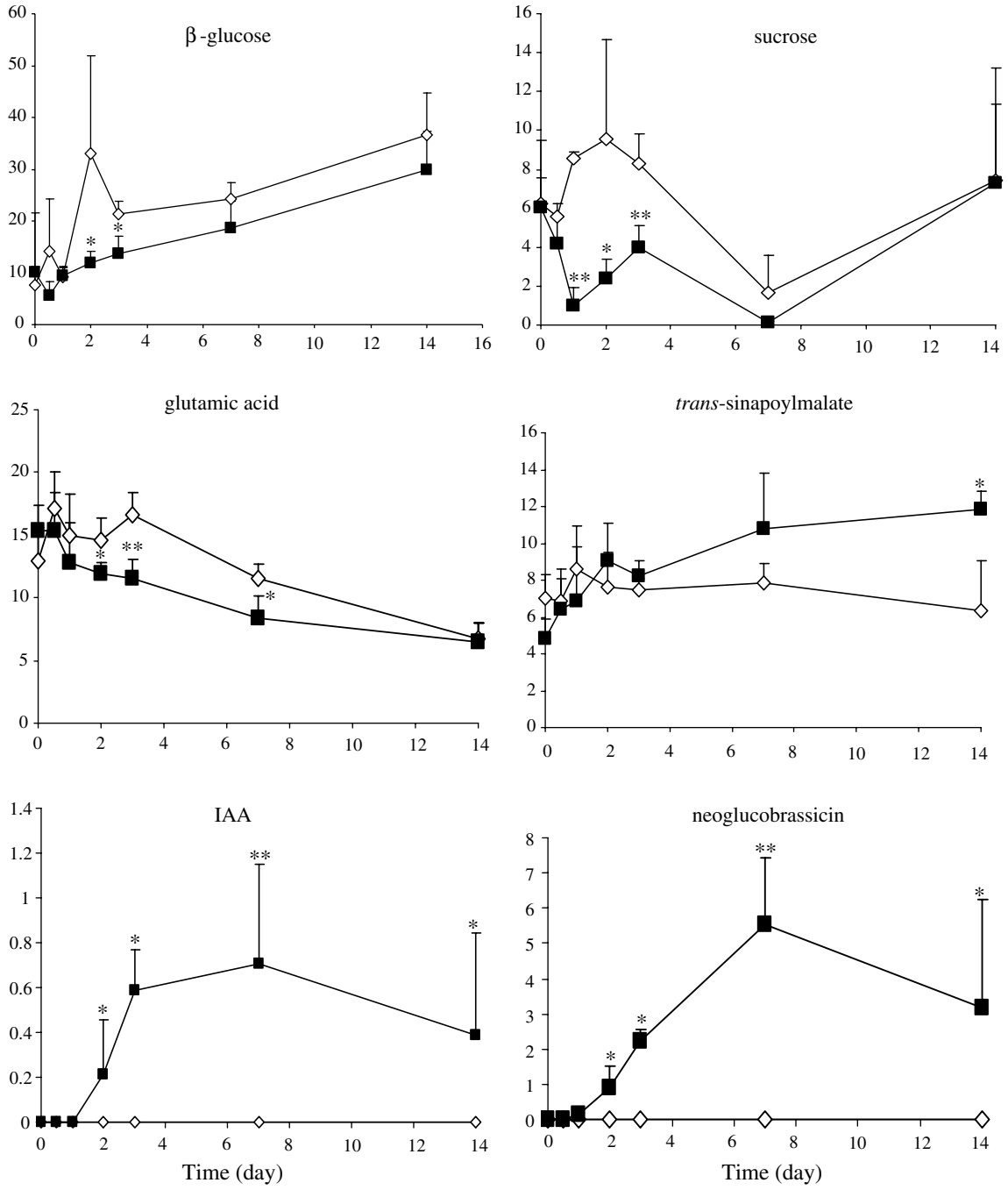


Fig. 6. Comparison of levels of several representative metabolites in *Brassica* changed after MJ treatment. *Y*-axis expresses the relative peak area of corresponding signals to that of internal standard (TSP). The projected signals onto *X*1-axis of 2D *J*-resolved NMR spectra were used for the integration, which appears mostly as a singlet. Control plants are shown as open diamond (◇) and MJ treated plants as solid box (■). *, MJ treated plants are significantly different from control plants ($p < 0.05$); **, MJ treated plants are significantly different from control plants ($p < 0.01$).

that myrosinase activation is part of the defence mechanism, the production of IAA may also be part of the defense response of plants. Stelmach et al. (1999) reported that JA treatment led to increased levels of endogenous IAA in *Bryonia dioica*, suggesting that octadecanoids produced as a result of external stimulation may act via/or in concert with IAA.

Malic acid conjugated with *trans*-hydrocinnamic acids such as *trans*-sinapoylmalate, *trans*-feruloylmalate and *trans*-coumaroylmalate are increased up to 14 days. Among the phenylpropanoids, the subgroup of hydroxycinnamic acids is very common in plants, e.g. ferulic-, sinapic-, caffeic and *p*-coumaric acid, usually occurring as esters of sugars, organic acids or amino acids (Mølgaard and Ravn, 1988). These phenylpropanoids are known to play an important role in cell wall extension. For example, feruloylmalate coupled to coniferyl alcohol was proposed as an intermediate that is transesterified to polysaccharides

in the cell wall (Rohde et al., 2004), forming a physical barrier around the site of the pathogen attacks hampering further spreading of the infection.

3. Conclusion

We showed that it is possible to detect differences in metabolite profiles between control and MJ treated *Brassica*by multivariate analysis of *J*-resolved 2D NMR spectra. Using this method we were able to analyze the metabolite changes produced by MJ treatment involving a wide range of compounds such as glucosinolates, hydroxycinnamates, sugars and amino acids. It was found that after MJ treatment, glucosinolates and hydroxycinnamates were increased in long term and glucose, sucrose and amino acids decreased relatively earlier time point. The production of indole metabolites such as neoglucobrassicin and IAA, and hydroxycinnamates may be important in defence responses in *Brassica*.

4. Experimental

4.1. Solvents and chemicals

Analytical grade MeOH was obtained from Merck Biosolve Ltd. (Valkenswaard, The Netherlands). CD_3OD (99.96%) and D_2O (99.00%) were purchased from Cambridge Isotope Laboratories Inc. (Miami, FL, USA) and NaOD was purchased from Cortec (Paris, France). MJ was obtained from Sigma Co. (St. Louis, MO).

4.2. Plants

Seeds of *Brassica rapa* var. *rapa* (Raapstelen, Groene Gewone) were germinated in soil in the greenhouse. Seedlings (10 cm high) were transferred into pots and grown for 8 weeks (25 °C, 50% humidity, 16 h light/8 h dark cycle).

4.3. Methyl jasmonate treatment

Four hundred micrograms of methyl jasmonate in 40% aq. EtOH (1 mg ml^{-1}) was topically applied on the surface of each leaf (8th–13th true leaves, four individual plants for each time point). Glass spatulas were used to spread the solution. In the case of control plants (three individual plants for each time point), 40% aq. EtOH was applied to the surface of the leaves. To avoid the effect of volatile MJ on control plants, MJ treatment was performed in a separate growth chamber under identical conditions as in the control chamber. After treatment, plants were harvested at 0 h, 12 h, 1 d, 2 d, 3 d, 7 d, 14 d and immediately frozen in liquid nitrogen and stored in −80 °C before extraction.

4.4. Extraction of plant material

The leaves were ground in liquid nitrogen with a mortar and a pestle and freeze-dried. Fifty milligrams of dried leaves were extracted with 1.5 ml of CD_3OD:D_2O (KH_2PO_4 buffer, pH 6.0, 1:1, v/v) containing 0.05% TSP (trimethyl silyl propionic acid sodium salt, w/v) as an internal standard, and sonicated for 10 min. To reduce risk of enzymatic conversion, CD_3OD was added first, followed by the D_2O (KH_2PO_4 buffer, pH 6.0). After centrifugation at 3000 rpm for 20 min, 800 μl of the supernatant was transferred to an NMR tube.

4.5. NMR measurements

^1H NMR and *J*-resolved spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR frequency of 400.13 MHz. CD_3OD was used as the internal lock. Each spectrum consisted of 128 scans, requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 90° (6.6 μs), and relaxation delay (RD) = 5.0 s. A presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zerofiltered to 32 K points. The window functions were optimized for the analysis. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, always using XWIN NMR (version 3.5, Bruker). Two dimensional *J*-resolved ^1H NMR spectra were acquired using 8 scans per 32 increments that were collected into 16 K data points, using spectral widths of 5.208 kHz in F2 (chemical shift axis) and 50 Hz in F1 (spin–spin coupling constant axis). A 1.0 s relaxation delay was employed, giving a total acquisition time of 14.52 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions prior to double complex FT. *J*-resolved spectra tilted by 45°, was symmetrized about F1, and then calibrated, always using XWIN NMR (version 3.5, Bruker). Data were exported as the 1D projection (F2 axis) of the 2D *J*-resolved spectra.

4.6. Data analysis

The ^1H NMR and the *J*-resolved projection spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to TSP and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ −0.40 to δ 10.00. A bin size of 0.04 ppm was used, which has shown in previous studies to give best results. The region of δ 4.7–5.0 was excluded from the analysis because of the residual signal of water and the region of citric acid, malic acid, and succinic acid in δ 2.8–2.5 was bucketed by 0.1 ppm in order to avoid the problems due to chemical shift changes in the spectra of these compounds dependent on their concentration. Principal component analyses (PCA) were per-

formed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

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