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Metabolic Characterization of *Brassica rapa* Leaves by NMR Spectroscopy

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The *Brassica* has been intensively studied due to the nutritional and beneficial effects. However, many species, varieties, and cultivars of this genus and the resulting large metabolic variation have been obstacles for systematic research of the plant. In order to overcome the problems posed by the biological variation, the metabolomic analysis of various cultivars of *Brassica rapa* was performed by NMR spectroscopy combined with multivariate data analysis. Discriminating metabolites in different cultivars and development stages were elucidated by diverse 2D-NMR techniques after sorting out different significant signals using ^{1}H NMR measurements and principal component analysis. Among the elucidated metabolites, several organic and amino acids, carbohydrates, adenine, indole acetic acid (IAA), phenylpropanoids, flavonoids, and glucosinolates were found to be the metabolites contributing to the differentiation between cultivars and age of *Brassica rapa*. On the basis of these results, the distribution of plant metabolites among different cultivars and development stages of *B. rapa* is discussed.

KEYWORDS: Metabolomic analysis; NMR; principal component analysis; *Brassica rapa*; cultivars; biological variation; developmental stages

INTRODUCTION

Brassica species constitute an important source of vegetable oils (*1, 2*) and proteins for human (*3*) and animal nutrition (*4*). Increased popularity of *Brassica* vegetables has resulted in a considerable increase in its production in Europe and the USA not only as a vegetable but also as an industrial source for seed oils and biofuel (*5–7*). The importance of *Brassica* as a food source is attributed to its high content of diverse health-promoting antioxidant metabolites such as phenolics, flavonoids, vitamins C and E (*8–10*), phenylpropanoids (*11*), and carotenoids (*12, 13*). Moreover, glucosinolates and their hydrolysis products, commonly found in the cruciferous (Brassicaceae family) including *Brassica* species, were found to possess antitumor activities (*14–19*). Epidemiological studies have shown protective effects of cruciferous vegetables, in particular against tumors of the lower bowel.

In addition to the commercial use of *Brassica*, its high level of genetic resemblance to *Arabidopsis* has made it an alternative model system in the field of plant physiology. Because it also occurs in the wild, it is considered an important plant model to study the interaction between plant and other organisms such as insects, fungi, or bacteria (*20–22*). In terms of metabolic expression some phenylpropanoids, flavonoids and glucosinolates have been assumed to play a role in defense of *Brassica* against other organisms (*11, 23, 24*).

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Although the *Brassica* genus has been intensively studied due to its nutritional and health benefits, the existence of numerous species, varieties, and cultivars results in a large metabolic variation which has constituted an obstacle for the systematic research of these plants. For instance, one of the major *Brassica* metabolite groups, the glucosinolates, shows a large variation among species, up to the point that there are many species-specific glucosinolates (*25, 26*). In the case of flavonoids, *Brassica* leaves have been reported to accumulate flavonols (quercetin, kaempferol, and isorhamnetin) and flavones (apigenin and luteolin) (*8*). Other species, such as *B. alba*, have been found to contain three flavonoids isolated from shoots, roots, and root exudates and identified as 3,5,6,7,8-pentahydroxy-4′-methoxyflavone, 2′,3′,4′,5′,6′-pentahydroxychalcone, and 3,5,6,7,8-pentahydroxyflavone together with apigenin from the shoots and roots (*27*). However, isorhamnetin-3,7-*O*-di-β-D-glucopyranoside was isolated only from the corolla of *B. rapa* flowers (*2*). Recently, isorhamnetin, kaempferol, and quercetin glycosides were also identified in turnip tops of *Brassica rapa* (*28*). Another important group of phenolic *Brassica* compounds, phenylpropanoids, shows a different metabolic profile depending on the species and tissues studied. In a recent study, a new phenylpropanoid, malate conjugated 5-hydroxyferulic acid, was identified in *Brassica rapa* leaves (*11*). In addition to the observed variation in the level of phenylpropanoids in different species, a variation in the types of tissue in a plant was also observed, e.g., a predominance of malate derivatives in leaves but choline

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forms in seeds (*29*). Furthermore, changes due to the attack of insects were reported (*20*).

In recent years, metabolomics has become prominent as a part of systems biology with the expectation that functional genomics will uncover unknown gene functions. Moreover, metabolomics is of interest in chemical classification of plants for chemotaxonomy and for following perturbations of living organisms by external factors. The term metabolome has been used to describe the observable chemical profile or fingerprint of the metabolites present in whole cells, tissues, or whole organisms (*30*). Any significant change brought about by conditions that affect living organisms are expected to show up in the profile of its metabolome. However, measuring the metabolome as an indicator for the biological interactions poses some problems, particularly the large natural biological variation in a species: the inability to comprehensively profile all the metabolomes due to their chemical complexity; also metabolomic profiling in crude extracts is not an easy task to perform since natural products display a wide range of structural diversity, and plants contain a large number of compounds with very different times of synthesis and quantities.

A metabolomics study can provide significant results if the metabolic changes in the target group exceed the biological variation of the control group. The metabolome is dynamic. Some metabolites have both spatial and temporal variation in plant species, and even under a given experimental condition, i.e., in a 1 or 2 week period, the metabolome of a plant can completely change (unpublished data). For example, it has been reported that the glucosinolate content in *Brassica* is dependent on several factors such as cultivar, age, seasonal, and environmental factors (temperature, soil type, and fertilizer application). Glucosinolates may even differ in quantity within the same organ during the day (*31–36*). Indeed, there are so many factors that affect the metabolome that, particularly in the case of *Brassica* metabolomics, there is an urgent need to investigate the influence of the most important factors such as species, varieties, cultivars, growing conditions, and age on the metabolic differentiation.

In this study, NMR spectroscopy coupled with multivariate data analysis was applied to *Brassica* metabolomics in order to investigate the metabolic differentiation of various cultivars and ages of *Brassica rapa* leaves. Four- and 6-week-old plants of four cultivars of *B. rapa* (Raapstelen, Witte Mei, Herfstraap and Oleifera) were examined for their metabolomic discrimination.

MATERIALS AND METHODS

Materials. Different *Brassica rapa* cultivars were submitted to uniform culture conditions. Seeds from registered cultivars including Raapstelen (Groene Gewone), Witte Mei (Witte Mei), Herfstraap (Goldana), and Oleifera were germinated in soil in the cold room (4 °C) for 2 days. The pots were placed in a box, provided with sufficient water, and covered. These were then transferred to the greenhouse and kept at 25 °C and 50–60% relative humidity with 16 h of daylight and 8 h of darkness per day. Seven-day-old seedlings were transferred to 10 cm diameter pots with substrate and placed in the same greenhouse and watered daily. Three individual plants were used as replicates from each cultivar. Four upper leaves from each plant were harvested 4 and 6 weeks after germination at around 13.00 h and plunged directly into liquid nitrogen before freeze-drying to avoid possible enzyme degradation. The samples were then ground to a fine powder with a pestle and mortar and stored in the cold room until analyzed.

Methods. *Extraction.* Freeze-dried plant material (50 mg) was transferred to a 2 mL microtube. A volume of 1.5 mL of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethylsilylpropionic acid sodium salt (w/w) (TSP) and methanol-d_4 (1:1) was added to the plant samples. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13000 rpm for 10 min. An aliquot of 0.8 mL was used for NMR analysis.

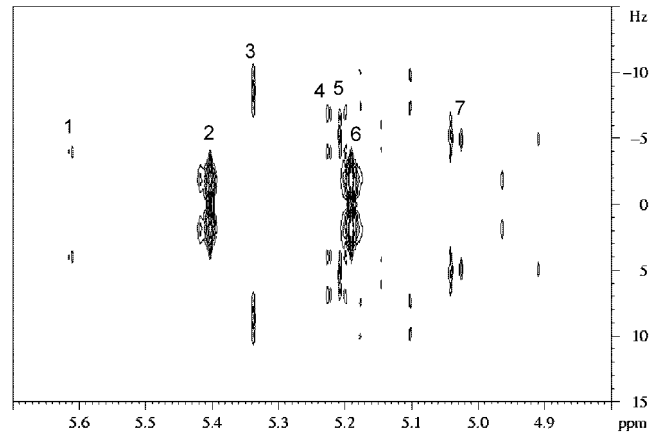

Figure 1. Two dimensional ^{1}H–^{1}H *J*-resolved spectrum of *Brassica rapa* Raapstelen upper leaves in the range of δ 4.8–δ 5.7: 1, anomeric protons of flavonoid glycosides; 2, sucrose; 3, H-5a of progoitrin; 4, H-2 of malic acid conjugated to *trans*-phenylpropanoids; 5, H-5b of progoitrin; 6, α-glucose; 7, anomeric proton of progoitrin.

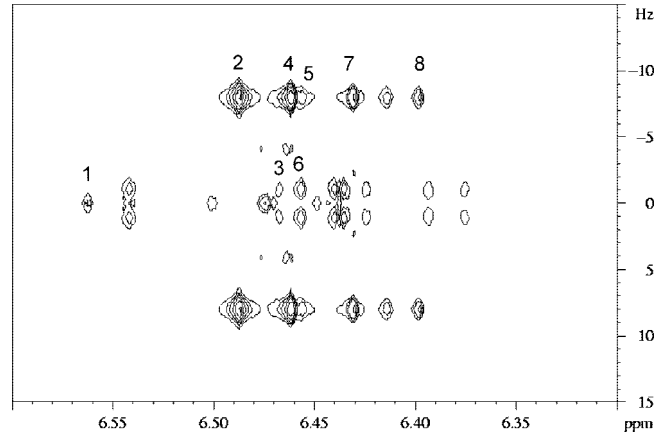

Figure 2. Two-dimensional ^{1}H–^{1}H *J*-resolved spectrum of *Brassica rapa* Raapstelen upper leaves in the range of δ 6.3–δ 6.6: 1, fumaric acid; 2, H-8′ of *trans*-sinapoyl malate; 3, H-6 of quercetin glycoside; 4, H-8′ of *trans*-feruloyl malate; 5, H-8′ of *trans*-coumaroyl malate; 6, H-6 of kaempferol glycoside; 7, H-8′ of *trans*-5-hydroxyferuloyl malate; 8, H-8′ of *trans*-caffeoyl malate.

NMR Measurements. ^{1}H NMR and 2D *J*-resolved spectra were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d_4 was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 μs), and relaxation delay (RD) = 1.5 s. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier-transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline-corrected and calibrated to TSP at 0.0 ppm, using XWIN NMR (version 3.5, Bruker). 2D *J*-resolved NMR spectra were acquired using 8 scans per 128 increments for F1 and 8K for F2 using spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). A 1.5 s relaxation delay was employed, giving a total acquisition time of 56 min. Data sets were zero-filled to 512 points in F1, and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. *J*-resolved spectra tilted by 45° were symmetrized about F1 and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H–^{1}H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC)

Table 1. ^{1}H Chemical Shifts (δ) and Coupling Constants (Hz) of *Brassica rapa* Metabolites Identified by References and Using 1D and 2D NMR Spectra (CD_3OD–KH_2PO_4 in D_2O (pH 6.0)

compound	chemical shifts and coupling constants
fatty acids (terminal CH_3)	0.91 (t, J = 7.5 Hz), 0.94 (t, J = 7.5 Hz)
valine	1.00 (d, J = 7.0 Hz), 1.05 (d, J = 7.0 Hz), 2.3 (m), 3.3 (d, J = 16.0 Hz)
threonine	1.32 (d, J = 7.0 Hz), 3.52 (d, J = 12.0 Hz), 4.23 (m)
lactic acid	1.36 (d, J = 7.0 Hz), 4.14 (d, J = 7.0 Hz)
alanine	1.48 (d, J = 7.4 Hz), 3.73 (q)
GABA	1.91 (t, J = 7.5 Hz), 2.35 (t, J = 7.5 Hz), 3.01 (t, J = 7.5 Hz)
acetic acid	1.94 (s)
glutamic acid	2.13 (m), 2.42 (m), 3.71 (dd, J = 6.8, 1.6 Hz)
glutamine	2.14 (m), 2.43 (td, J = 16.5, 7.4 Hz)
succinic acid	2.53 (s)
malic acid (free)	2.58 (dd, J = 16.0, 7.4 Hz), 2.76 (dd, J = 16.0, 4.6 Hz), 4.32 (dd, J = 7.2, 4.0 Hz)
malic acid conjugated to *cis*-phenylpropanoids	2.65 (dd, J = 16.0, 11.0 Hz), 2.83 (dd, J = 16.0, 3.0 Hz), 5.19 (dd, J = 11.0, 3.0 Hz)
malic acid conjugated with *trans*-phenylpropanoids	2.7 (dd, J = 16.0, 12.0 Hz), 2.85 (dd, J = 16.0, 3.0 Hz), 5.22 (dd, J = 11.0, 3.0 Hz)
choline	3.24 (s)
fructose or sucrose	4.16 (d, J = 7.8 Hz)
sucrose	5.4 (d, J = 3.6 Hz)
β-glucose	4.58 (d, J = 7.8 Hz)
α-glucose	5.18 (d, J = 3.7 Hz)
progoitrin	2.87 (dd, J = 16.0, 10.0 Hz), 4.63 (m), 5.21 (dt, J = 11.0, 2.0 Hz), 5.34 (dt, J = 16.0, 2.0 Hz), 5.96 (m)
neoglucobrassicin	4.09 (s, MeO), 7.17 (t, J = 7.8 Hz), 7.30 (t, J = 7.8 Hz), 7.49 (d, J = 7.8 Hz), 7.48 (s), 7.73 (d, J = 7.8)
indoleacetic acid	3.25 (d, J = 16.0 Hz), 3.39 (d, J = 16.0 Hz), 7.12 (s), 7.13 (t, J = 7.8 Hz), 7.21 (t, J = 7.8 Hz), 7.47 (d, J = 7.8 Hz), 7.72 (d, J = 7.8 Hz)
adenine	8.2 (s), 8.21 (s)
fumaric acid	6.56 (s)
formic acid	8.46 (s)
kaempferol analogues	6.46 (d, J = 2.1 Hz), 6.77 (d, J = 2.0 Hz), 7.00 (d, J = 8.8 Hz), 8.07 (d, J = 8.8 Hz)
quercetin analogues	6.47 (d, J = 2.1 Hz), 6.77 (d, J = 2.0 Hz), 6.9 (d, J = 8.8 Hz), 7.54 (dd, J = 6.5, 3.0 Hz), 7.82 (d, J = 2.0 Hz)
cis-sinapoyl malate	5.94 (d, J = 13.0 Hz), 6.95 (d, J = 13.0 Hz), 7.12 (s)
trans-sinapoyl malate	6.49 (d, J = 16.0 Hz), 6.97 (s), 7.65 (d, J = 16.0 Hz)
cis-caffeoyl malate	5.93 (d, J = 13.0 Hz), 6.84 (d, J = 8.8 Hz), 6.94 (d, J = 13.0 Hz), 7.06 (dd, J = 8.4, 2.0 Hz), 7.84 (d, J = 2.0 Hz)
trans-caffeoyl malate	6.4 (d, J = 16.0 Hz), 6.83 (d, J = 8.8 Hz), 7.13 (dd, J = 8.4, 2.0 Hz), 7.15 (d, J = 2.0 Hz), 7.61 (d, J = 16.0 Hz)
cis-feruloyl malate	5.97 (d, J = 13.0 Hz), 6.84 (d, J = 8.8 Hz), 6.94 (d, J = 13.0 Hz), 7.13 (dd, J = 8.4, 2.0 Hz), 7.83 (d, J = 2.0 Hz)
trans-feruloyl malate	6.47 (d, J = 16.0 Hz), 6.87 (d, J = 8.4 Hz), 7.06 (dd, J = 8.4, 2.3 Hz), 7.26 (d, J = 2.0 Hz), 7.65 (d, J = 16.0)
cis-coumaroyl malate	5.97 (d, J = 13.0 Hz), 6.95 (d, J = 13.0 Hz), 7.00 (bd, J = 9.2 Hz), 7.89 (bd, J = 9.2 Hz)
trans-coumaroyl malate	6.46 (d, J = 16.0 Hz), 6.84 (bd, J = 8.8 Hz), 7.57 (bd, J = 9.2 Hz), 7.66 (d, J = 16.0 Hz)
cis-5-hydroxyferuloyl malate	5.94 (d, J = 13.0 Hz), 6.95 (d, J = 13.0 Hz), 7.13 (d, J = 2.0 Hz), 7.51 (d, J = 2.0 Hz)
trans-5-hydroxyferuloyl malate	6.43 (d, J = 16.0 Hz), 6.84 (d, J = 2.0 Hz), 6.87 (d, J = 2.0 Hz), 7.59 (d, J = 16.0 Hz)

spectra were recorded on a 600 MHz Bruker DMX-600 spectrometer (Bruker). The COSY spectra were acquired with 1.0 s relaxation delay, 6361 Hz spectral width in both dimensions. Window function for COSY spectra was sine-bell (SSB = 0). The HSQC spectra were obtained with 1.0 s relaxation delay, 6361 Hz spectral width in F2 and 27 164 Hz in F1. Qsine (SSB = 2.0) was used for the window function of the HSQC. The HMBC spectra were recorded with the same parameters as the HSQC spectrum, except for 30 183 Hz of spectral width in F2. The optimized coupling constants for HSQC and HMBC were 145 and 8 Hz, respectively.

Data Analysis. ^{1}H NMR spectra were automatically reduced to ASCII (v. 3.7, Bruker Biospin). Intensities were scaled to TSP and reduced to integrated regions of equal width (0.04) corresponding to the region of δ 0.4–δ 10.0. The region of 4.8–4.9 was excluded from the analysis because of the residual signal of water. Principal component analysis (PCA) was performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

RESULTS AND DISCUSSION

Four cultivars of *Brassica rapa* (Raapstelen, Herfstraap, Witte Mei, and Oleifera) of two different ages, 4 and 6 weeks old in this case, were analyzed by NMR to evaluate the effect of cultivar type and age on metabolic variation. Of the possible analytical techniques generally used in metabolomics, MS and NMR-based methods are usually acknowledged to be the optimum choice for a metabolic study. NMR, particularly ^{1}H NMR, has advantages unsurpassed by other methods such as the ease of simultaneous detection of diverse groups of metabolites in a relatively short measuring time. Here, the study was performed on the basis of known compounds recognized in NMR spectra of previous studies. Therefore, the influence of cultivar and age can easily be examined with respect to these compounds. In spite of the relative ease of NMR method

implementation, signal overlapping does constitute an obstacle to identifying each metabolite in the extract. As shown in **Figures 1** and **2**, this problem was partly solved by acquiring 2D ^{1}H–^{1}H *J*-resolved NMR spectra, which provided additional information and the splitting pattern of each signal with the exact coupling constant. In fact, the *J*-resolved spectra together with correlation 2D-NMR spectra including COSY, HMQC, and HMBC considerably increased the number of identified metabolites beyond that from a 1D ^{1}H NMR spectra.

In the amino acid region (δ 0.8–δ 4.0), alanine, glutamic acid, glutamine, threonine, and valine were identified (**Table 1**). Organic acids such as acetic, formic, fumaric, lactic, malic, succinic, and γ-aminobutyric acid (GABA) as well as choline and signals of the terminal CH_3 of fatty acids or lipids were identified (**Table 1**). For sugars, the anomeric proton of β-glucose at δ 4.58 (d, $J = 7.8$ Hz), α-glucose at δ 5.18 (d, $J = 3.7$ Hz), sucrose at δ 5.4 (d, $J = 3.6$ Hz), and fructose moiety with sucrose at δ 4.16 (d, $J = 7.8$ Hz) were assigned. In this region, the anomeric signals at δ 4.97, δ 5.03, and δ 5.05 with the characteristic large coupling constants ($J = 9.8$–10.0 Hz) different from those of common anomeric sugar protons (1.0–8.0 Hz) indicated the presence of the glucose moiety of aliphatic or aromatic glucosinolates (*37*). Of these glucosinolates, progoitrin was identified by 2D NMR (**Figure 1**). The anomeric proton of progoitrin was detected at δ 5.03 and correlated in HMBC with the carbon at δ 163.3. The signal of H-2 of progoitrin was detected at δ 2.87 (dd, $J = 16.0$, 10.0 Hz), H-3 at δ 4.63 (m), H-4 at δ 5.96 (m), H-5a at δ 5.34 (dt, $J = 16.0$, 2.0 Hz), and H-5b at δ 5.21 (dt, $J = 11.0$, 2.0 Hz). Correlation between H-3 and the carbon at δ 118.0 in HMBC confirmed the position of OH group. In the COSY spectrum H-2, H-3, H-4, and H-5 were correlated with each other. These signals correlated with carbons in HSQC at δ 68.2, δ 94.0, δ 115.0, and δ 114.0, respectively. Neoglucobrassicin, an indolic glucosinate, was identified by using *J*-resolved and COSY spectra. Additionally, indoleacetic acid (IAA) was detected as a minor compound in the plant mixture (**Table 1**). Both neoglucobrassicin and indole acetic acid were found to increase in *B. rapa* after treatment with methyl jasmonate (*37*).

The 2D *J*-resolved spectrum of the plant extract showed characteristic double–doublet signals at δ 5.22 (dd, $J = 11.0$, 3.0 Hz) and at δ 5.19 (dd, $J = 11.0$, 3.0 Hz). These signals were assigned as the H-2 of malic acid conjugated with phenylpropanoids, based on the correlation in the COSY spectrum with the signals of H-3 of malic acid at δ 2.70 and δ 2.85 (dd, $J = 16.0$, 12.0, and 16.0, 3.0 Hz conjugation with -phenylpropanoids) and at δ 2.65 and δ 2.83 (dd, $J = 16.0$, 11.0 Hz and 16.0, 3.0 Hz conjugation with *cis* forms).

In the aromatic region, the presence of five major doublets with the same coupling constants (d, $J = 16.0$ Hz) in the range of δ 6.39–δ 6.49 indicated the presence of the *trans* olefinic protons of the phenylpropanoids (**Figure 2**). This was confirmed by the fact that H-8′ of the phenylpropanoids correlated with the H-7′ protons at δ 7.59–δ 7.66 in the COSY spectrum and with the carboxyl group at δ 171.3 in the HMBC spectrum. Five *trans*-phenylpropanoids and two *cis* forms were elucidated by two dimensional NMR. However, the *cis*-phenylpropanoids are believed to be artifacts of *trans*-phenylpropanoids produced during the sample extraction (*37*, *38*). These compounds were already detected in methyl jasmonate treated plants, together with another three *cis*-phenylpropanoids (*37*).

A flavonoid was detected and identified as kaempferol glycoside. The signals at δ 6.46 and δ 6.77 correlated with each other in the COSY spectrum with a *meta* coupling constant ($J = 2.0$ Hz) were assigned to be H-6 and H-8 of kaempferol glycoside. The presence of the flavonoid was confirmed by the HSQC spectrum in which the carbons of H-6 and H-8 showed upfield shifts (97.9 and 93.2 ppm), respectively. The correlation between the signals at δ 7.00 (H-2′ H-6′, d, $J = 8.8$ Hz) and δ 8.07 (H-3′ H-5′, d, $J = 8.8$ Hz) led to the elucidation of the B-ring protons of kaempferol. The downfield shift of H-8 showed the attachment of glucose at the C-7 position. Another flavonoid, quercetin glycoside, was also detected. A signal at δ 6.47 of H-6 (d, $J = 2.0$ Hz) was correlated in the COSY spectrum with the signal at δ 6.77 of H-8 (d, $J = 2.0$ Hz) and a signal at δ 6.90 of H-5′ (d, $J = 8.8$ Hz) with one at δ 7.54 of H-6′ (dd, $J = 6.5$, 3.0 Hz). The signal of H-2′ was detected at δ 7.82 (d, $J = 2.0$ Hz). An anomeric proton of β-glucose in flavonoid glycosides was detected in the *Brassica* extracts at δ 5.61 (d, $J = 7.4$). This signal correlated with the carbon at δ 166.0 and 179.6, respectively.

PCA was done on the basis of small integrated regions on the processed raw data. The separation seen in the PCA is explained in terms of the identified compounds. Six-week-old *Brassica* plant leaves showed a higher level of carbohydrates but less organic and amino acids compared to 4-week-old samples. In the aromatic region, indoleacetic acid (IAA) and

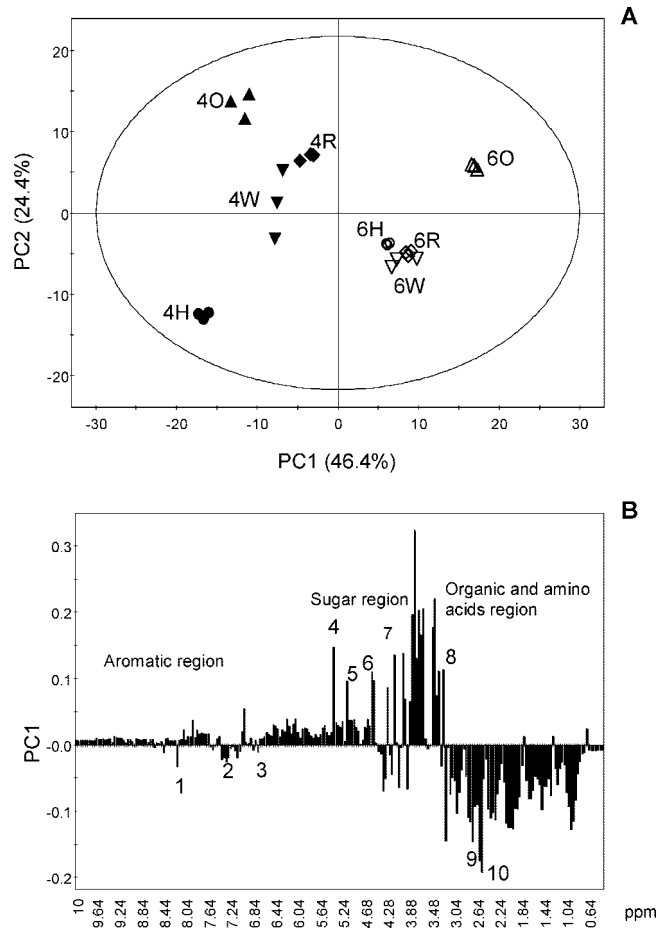

Figure 3. Score (PC1 vs PC2, **A**) and loading (PC1, **B**) plot of PCA results obtained from ^{1}H NMR spectra of four cultivars of *Brassica rapa* upper leaves of 4 and 6 weeks old. 4H: 4-week-old Herfstraap (•); 4O: 4-week-old Oleifera (▲); 4R: 4-week-old Raapstelen (♦); 4W: 4-week-old Witte Mei (▼); 6H: 6-week-old Herfstraap (○); 6O: 6-week-old Oleifera (Δ); 6R: 6-week-old Raapstelen (◇); 6W: 6-week-old Witte Mei (▽). 1, adenine; 2, indoleacetic acid (IAA); 3, flavonoid glycoside; 4, sucrose; 5, α-glucose; 6, β-glucose; 7, fructose moiety of sucrose; 8, choline; 9, malic acid; 10, succinic acid.

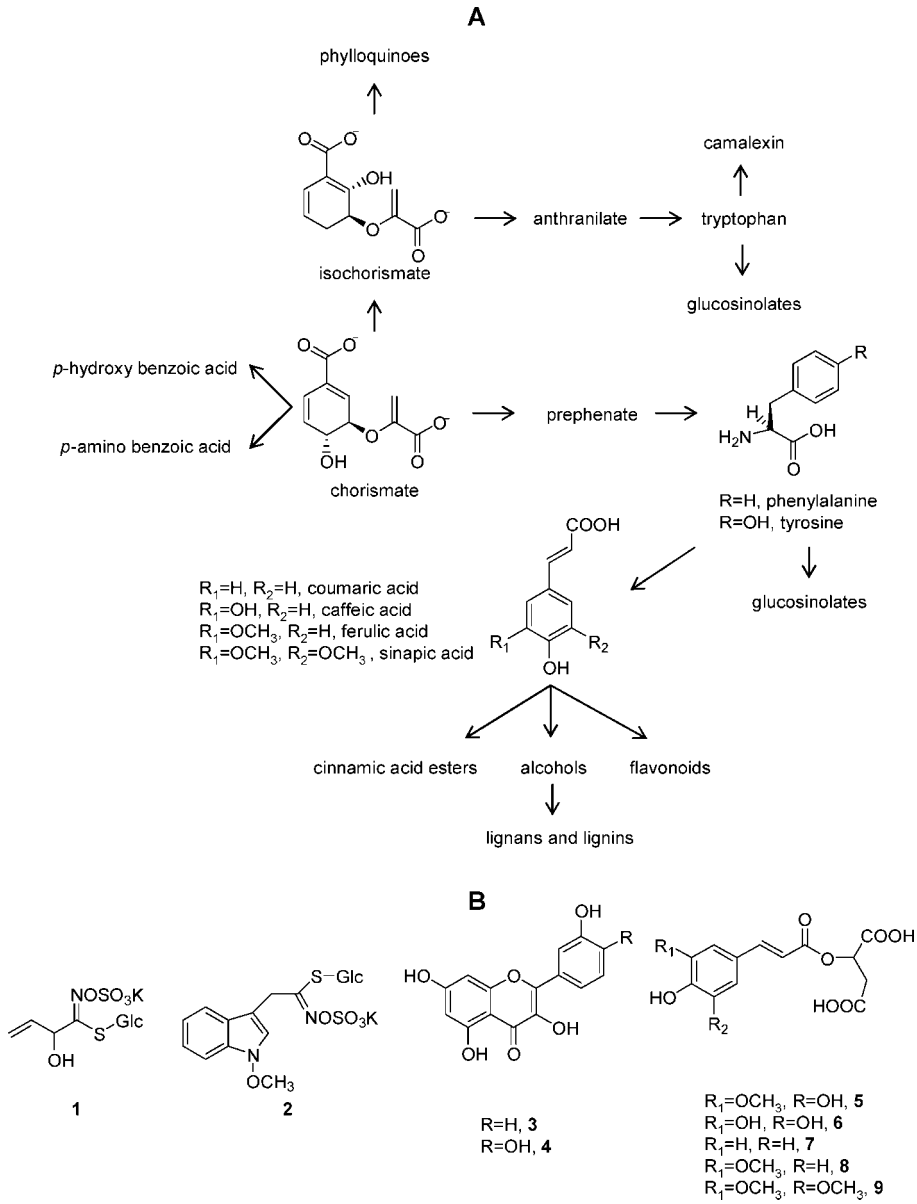

Figure 4. Cinnamic acid derivatives pathways for formation of secondary metabolites in plants (**A**) and structure of some detected secondary metabolites in *Brassica rapa* extract (**B**): 1, progoitrin; 2, neoglucobrassicin; 3, kaempferol; 4, quercetin; 5, 5-hydroxyferuloyl malate; 6, caffeoyl malate; 7, coumaroyl malate; 8, feruloyl malate; 9, sinapoyl malate.

flavonoid signals at δ 7.1–δ 7.6 and δ 6.76 were found to be higher in 4-week-old samples (**Figure 3A**,**B**) The two increased singlets at δ 8.2 in the 4-week-old samples were assigned to adenine by comparison with the spectra of a reference compound. Other aromatic signals including phenylpropanoids and glucosinolates were higher in the older plants. The higher level of carbohydrates observed in the upper leaves of 6-week-old leaves may be attributed to the increase in all leaves total surface area with age, which in turn increases the rate of photosynthesis, thus producing a greater accumulation of sugars.

Leaves contribute actively to plant fitness so they are expected to contain a higher concentration of compounds such as phenylpropanoids or glucosinolates associated to defense mechanisms. The high concentration of these compounds in the top leaves is thought to reflect the need of these tissues to increase their defensive potential (*35*). The buildup of these secondary metabolites which are derived from the organic and amino acids may explain the decrease of the latter observed in 6-week-old plants. The differences in flavonoid and phenylpropanoids levels at the different developmental stages might be due to a different channeling of the phenylpropanoid precursors (cinnamic acid derivatives) (**Figure 4A**,**B**). In *Arabidopsis*, for example, the accumulation of one or more flavonoids correlates with the decrease in level of cinnamic acid derivatives (*39*). Indoleacetic acid (IAA) is an important plant hormone contributing to a variety of developmental processes (*37*), and the higher concentration of adenine in 4-week-old plants might be due to a higher rate of metabolic activity in younger plants.

The glucosinolate content were reported to increase rapidly during the first 40 days after planting, reaching a maximum at 50 days. Thus, 6-week-old leaves showed a higher content of glucosinolates than 4-week-old leaves (*40*).

The PCA of 4-week-old plants alone showed a better separation of Herfstraap and Witte Mei leaves from those of Oleifera and Raapstelen than that obtained when both age groups were combined (**Figures 3A** and **5A**). The loading plot of PC1 revealed that Herfstraap and Witte Mei were well separated by the higher content of all detected amino acids, organic acids, glucosinolates, phenylpropanoids, flavonoids, and adenine from Oleifera and Raapstelen, which were separated by free malic

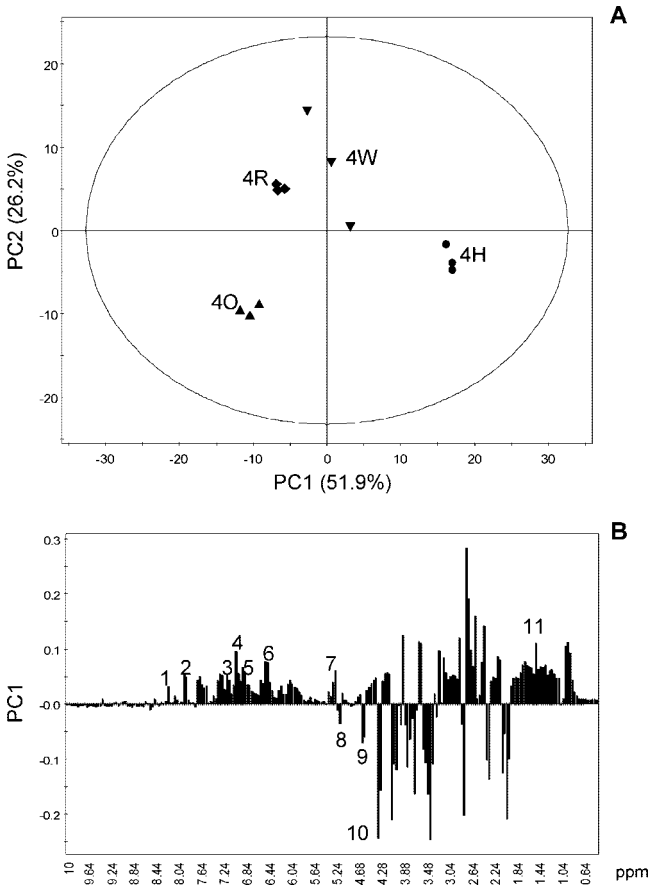

Figure 5. Score (PC1 vs PC2, **A**) and loading (PC1, **B**) plot of PCA results obtained from ^{1}H NMR spectra of four cultivars of *Brassica rapa* upper leaves of 4 weeks old. 4H: 4-week-old Herfstraap (•); 4O: 4-week-old Oleifera (▲); 4R: 4-week-old Raapstelen (♦); 4W: 4-week-old Witte Mei (▼). 1, adenine; 2, coumaroyl malate; 3, neoglucobrassicin; 4, sinapoyl malate; 5, flavonoid glycoside; 6, feruloyl and 5-hydroxyferuloyl malate; 7, progoitrin; 8, α-glucose; 9, β-glucose; 10, malic acid; 11, alanine.

acid, glucose, and choline (**Figure 5B**, Table 1 of the Supporting Information). PCA of 6-week-old plants separated all cultivars evaluated (**Figure 6A**). Herfstraap was separated by a higher content of amino and organic acids, glucosinolates, phenylpropanoids, and flavonoids, while Oleifera was found to have a higher content of carbohydrates (glucose, sucrose) and choline (**Figure 6B**). Witte Mei and Raapstelen were separated from Oleifera and Herfstraap by a higher content of sucrose, threonine, neoglucobrassicin, and *cis* form of phenylpropanoids (**Figure 6C**).

Both 4- and 6-week-old Herfstraap and Witte Mei *Brassica* leaves showed a higher concentration of glucosinolates, while Oleifera showed the lowest concentration among the studied cultivars. These results are in agreement with the HPLC quantitation of glucosinolates in 4- and 6-week-old plants (unpublished data). The characteristic low level of glucosinolates of the Oleifera cultivar might be the reason for its ease of the transformation of *Brassica* with *Agrobacterium* (*41*). In addition, significant variations of the aromatic signals were observed among the cultivars evaluated in this study; Oleifera showing significantly less phenolic compounds than other cultivars. These results are in accordance with previous reports (*41*).

This study shows that cultivars and development stage of the plant are important factors for the metabolome. The trends for development are similar for the four cultivars and in part might

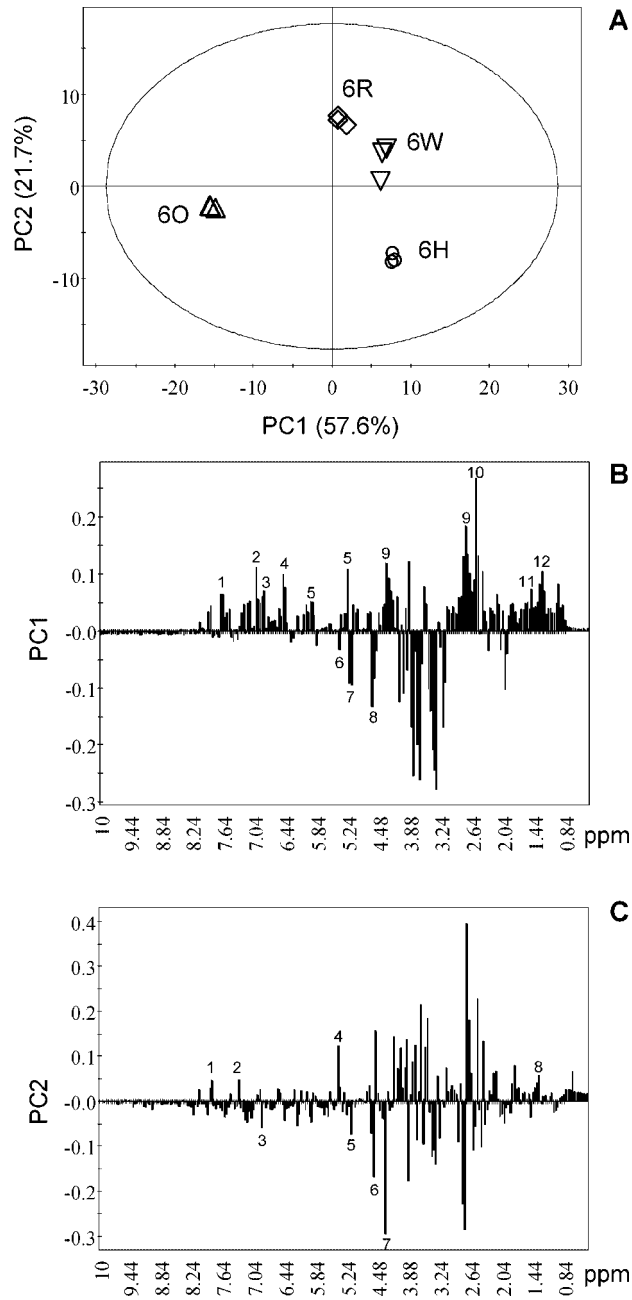

Figure 6. Score (PC1 vs PC2, **A**) and loadings (PC1, **B** and **C**) plot of PCA results obtained from ^{1}H NMR spectra of four cultivars of *Brassica rapa* upper leaves of 6 weeks old. 6H: 6-week-old Herfstraap (○); 6O: 6-week-old Oleifera (Δ); 6R: 6-week-old Raapstelen (◇); 6W: 6-week-old Witte Mei (▽). Assignments in (**B**): 1, coumaroyl malate; 2, sinapoyl malate; 3, flavonoid glycoside; 4, feruloyl and 5-hydroxyferuloyl malate; 5, progoitrin; 6, sucrose; 7, α-glucose; 8, β-glucose; 9, malic acid; 10, succinic acid; 11, alanine; 12, threonine. Assignments in (**C**): 1, *cis*-caffeoyl and feruloyl malate; 2, neoglucobrassicin; 3, *trans*-feruloyl malate; 4, sucrose; 5, α-glucose; 6, β-glucose; 7, malic acid; 8, threonine.

be due to changes in channeling of precursors. A further detailed time course of the secondary metabolite production would be of interest to learn more about this phenomenon, which is for example of interest in connection with the time of harvesting and the quality of the vegetable, e.g., in terms of taste and the presence of health promoting compounds. The pattern of metabolic variation among cultivars may provide an opportunity to select those suitable for further studies including plant–fungi or plant–insect interaction.

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Supporting Information Available: Table of mean and standard deviations of selected metabolites. This material is available free of charge via the Internet at http://pubs.acs.org.

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