Metabolomic changes of Brassica rapa under biotic stress

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Chapter 7

Metabolic assessment of *Brassica rapa* leaves treated with jasmonic and salicylic acid

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Abstract

The effect of two signal molecules, jasmonic acid (JA) and salicylic acid (SA), on the *Brassica rapa* var. *rapa* (Raapstelen) metabolome was investigated using nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC). The data were analyzed by principal component analysis (PCA). With NMR spectroscopy only signals of one aliphatic glucosinolate (progoitrin) and another indole glucosinolate (neoglucobrassicin) were detected due to the low sensitivity of NMR and overlapping of signals of glucosinolates with that of other secondary metabolites. So targeted analysis using HPLC was used to identify the individual glucosinolates in control and treated plants. Different patterns of accumulation between JA and SA treated plants with regard to primary and secondary metabolites were observed. Fatty acids, amino acids (alanine, threonine, valine and GABA), organic acid (succinic and fumaric acid), adenine, steroids, phenylpropanoids (sinapoyl-, coumaroyl- and 5-hydroxyferuloyl malate), flavonoids (kaempferol and quercetin), aliphatic glucosinolates (gluconapin, progoitrin, and gluconapoleiferin), indole glucosinolates (glucobrassicin, 4-hydroxy-glucobrassicin and neoglucobrassicin), benzoyl glucosinolates (gluconasturtiin and sinalbin) and indole acetic acid (IAA) significantly increased in JA treated plants. SA treated plants were characterized by high concentrations of fatty acids, amino acids (alanine, threonine and GABA), adenine, steroids, flavonoids (kaempferol), aliphatic glucosinolates (gluconapin, glucobrassicanapin), indole glucosinolates (neoglucobrassicin) and benzoyl glucosinolates (gluconasturtiin and sinalbin). JA and SA treated plants were characterized by lower concentrations of sucrose, choline and malic acid.

There is an immense effect of signal molecules on expression of many metabolites. JA and SA act more prominently and powerfully on glucosinolates than IAA, phenylpropanoids and flavonoids.

Keywords: *Brassica rapa*, salicylic acid, jasmonic acid, metabolomics, nuclear magnetic resonance spectroscopy, high performance liquid chromatography

7.1 Introduction

JA acts as a secondary messenger in the signal transduction events in cells. But also acts as an external signal compound, JA and its volatile methyl ester MJ, have inhibitory and promotory effects, often similar to the effect of abscisic acid on many physiological processes in the plant (Sembdner and Parthier, 1993). Among these effects is the induction of various biosynthetic pathways associated with response to wounding, herbivory and infection with microorganisms (Doughty *et al*., 1991; Bodnaryk, 1992; Sembdner and Parthier, 1992; Widarto *et al*., 2006). The jasmonates are key signal compounds in the elicitation process leading to *de novo* transcription and translation and, finally, to the biosynthesis of secondary metabolites in plants (Gundlach *et al*., 1992). Induction by MJ is not restricted to one type of secondary metabolites but to a wide spectrum of low molecular weight substances ranging from flavonoids, guaianoides, terpenoids, anthraquinones to various classes of alkaloids (Gundlach *et al*., 1992). Treatment of roots of red clover seedlings (*Trifolium pratense*) with JA induced formation of four hydroxycinnamic acid amide conjugates with aromatic amino acids (Tebayashi *et al*., 2000). MJ was found to induce coumaroyl conjugates (*N*-(*E*)-4-coumaroylputrescine and *N*-(*E*)-4-coumaroylagmatine) in barley leaves (Lee *et al*., 1997). Treatment of soybean seedlings with MJ enhanced the accumulation of anthocyanins (Franceschi and Grimes, 1991). MJ treated *Arabidopsis* showed induced accumulation of flavonoids (kaempferol and quercetin glucosides), sinapoyl malate and tryptophan (Hendrawati *et al*., 2006). *Brassica rapa* treated with MJ showed induced accumulation of phenylpropanoids (sinapoyl-, coumaroyl- caffeoyl-, feruloyl malate) and indole acetic acid (IAA) (Liang *et al*., 2006b).

Cruciferous plants produce a special class of metabolites, the glucosinolates, for different purposes, such as to protect themselves from herbivore attack and pathogens (Blaževic and Mastelić, 2009). Several studies were focused on the glucosinolate profiling after treatment with JA and MJ of *Brassica* (Bodnaryk, 1994; Doughty *et al*., 1995; Ludwig-Müller *et al*., 1997; van Dam *et al*., 2003; Loivamäki *et al*., 2004; Liang *et al*., 2006b) or *Arabidopsis* (Brader *et al*., 2001; Mikkelsen *et al*., 2003; Hendrawati *et al*., 2006). Treatment of different species of *Brassica* with JA or MJ enhanced the accumulation of specific indole glucosinolates in cotyledons and leaves (Bodnaryk, 1994). MJ sprayed *B. napus* accumulated progoitrin,

glucobrassicanapin, glucobrassicin and neoglucobrassicin together with benzoyl glucosinolate (gluconasturtiin) after 7d from treatment (Doughty *et al*., 1995).

Salicylic acid (SA) is an ubiquitous plant phenolic involved in many plant physiological processes, including heat production, floral induction, control of stomatal opening, ion uptake and inhibition of ethylene synthesis (Raskin, 1992). There is ample evidence that SA is a signal molecule in the sequence of metabolic events leading to the expression of systemic acquired resistance to plant pathogens (Yalpani and Raskin, 1993). Exogenous application of SA or its analogues, acetyl-salicylic acid and 2,6-dihydroxybenzoic acid results in the accumulation of pathogenesis-related proteins (PR), thought to have a function in disease resistance in many species (Malamy *et al*., 1990). SA induces the biosynthesis of secondary metabolites in plant cell cultures such as indole alkaloids in *Cathranthus roseus* (Zhao *et al*., 2001) and sesquiterpenes in both suspension cell and transformed root cultures of the *Hyoscyamus muticus* (Mehmetoglu and Curtis, 1997). Phenylpropanoids (caffeic-, *p*-coumaric-, ferulic- and sinapic acids) increased significantly after postharvest application of SA to grape berries with a maximum accumulation appearing 1d after treatment (Chen *et al*., 2006). *Brassica oleracea* showed higher concentrations of anthocyanins after SA treatment (Cole, 1996). Salicylic acid enhances the production of flavonoids (jaceosidin and syringin) which is accompanied with induction of the related phenylpropanoids biosynthetic enzymes in *Saussurea medusa* cell cultures (Yu *et al*., 2006). SA treated *Brassica campestris* ssp. *pekinensis* showed enhanced accumulation of indole glucosinolates dominated by neoglucobrassicin and 4-methoxy-glucobrassicin in treated roots and also in leaves together with gluconapin and gluconasturtiin (Ludwig-Muller *et al*., 1997). In *B. napus*, gluconapin, glucobrassicin and 4-methoxy-glucobrassicin increased after SA treatment (Kiddle *et al*., 1994; 2001).

Few studies were carried out to test the effect of methyl jasmonate, jasmonic acid and salicylic acid on the metabolome in *Brassica* species. None of them has considered the effect of the treatments on the entire metabolome of the plants beyond the glucosinolate content. Metabolomic studies are always considered to be complex due to the large number of metabolites involved. Though it is almost impossible for one single analytical method to provide information about all the metabolites in plants (Verpoorte *et al*., 2008), nuclear magnetic resonance spectroscopy (NMR) constitutes an optimum choice for the first step of a metabolomic study from a macroscopic

viewpoint. It is a non-destructive method and can simultaneously detect all proton-bearing compounds such as phenolics, carbohydrates, amino acids, fatty acids, amines, esters, lipids etc., in a short time (Pauli *et al*., 2005; Choi *et al*., 2006; Verpoorte *et al*., 2007, 2008; Abdel-Farid *et al*., 2007; Jahangir *et al*., 2008a). Although ^{1}H-NMR is rather insensitive compared to other methods such as mass spectrometry (MS), it has the advantage of allowing the detection of diverse groups of plant metabolites in a single run, thus motivating researchers to use it as the macroscopic approach for metabolomics. In spite of the importance of NMR as a tool for measuring the plant metabolome, other techniques such as HPLC should be used besides NMR for a detailed analysis of special groups of compounds that NMR can not detect because of the low sensitivity of the method, or overlapping of signals such as glucosinolates.

Considering that the signal molecules like JA or SA are involved in plant defense mechanisms, we may expect that treating plants with one of these signal compounds may alter not only glucosinolates but also the whole metabolome including other secondary metabolites like phenylpropanoids and flavonoids. In this study, NMR spectroscopy and HPLC combined with multivariate data analysis were used to explore the effects of JA and SA on the metabolome of *B. rapa*.

7.2 Material and Methods

7.2.1 Plant material

The seeds of *Brassica rapa* var. *rapa*, Raapstelen were obtained from the Department of Plant Ecology, Leiden University (Leiden, The Netherlands). Seeds were germinated in soil and placed in a cold room (4 ^{o}C) for two days in the dark and in a closed container. The seedlings were then transferred to the greenhouse and grown under controlled conditions, at 25 ºC, 50-60% humidity and 16h light/8h dark cycles. Each seven-day old seedling was transferred to a 10 cm-diameter pot containing substrate and grown in the same controlled room.

7.2.2 Treatment with jasmonic and salicylic acid

This experiment was carried out on six week-old *Brassica* plants, homogenized plants were selected for JA and SA treatment. Fourth leaves were treated with a 5mM solution of JA or SA in an aqueous solution containing 0.1% Triton-X100 (Ludwig-Müller *et al*., 1997; van Dam *et al*., 2003).

Controls were treated with 0.1% Triton-X100 only. Local (fourth) and systemic (sixth) leaves were harvested after 1, 7 and 14d from treatments. Leaves were immediately transferred to a vessel containing liquid nitrogen, ground under liquid nitrogen and then freeze-dried.

7.2.3 Extraction and NMR spectra measurements

Extraction and NMR spectra measurements were carried out according to our previous work described in **Chapter 3** (Abdel-Farid *et al*., 2007).

7.2.4 Glucosinolate analysis

Extraction was performed using the method described by Font *et al*. (2005) and Padilla *et al*. (2007). Details about the glucosinolates analysis are described in **Chapter 5**. Four replicates were analyzed for each sample.

7.2.5 Statistical analysis

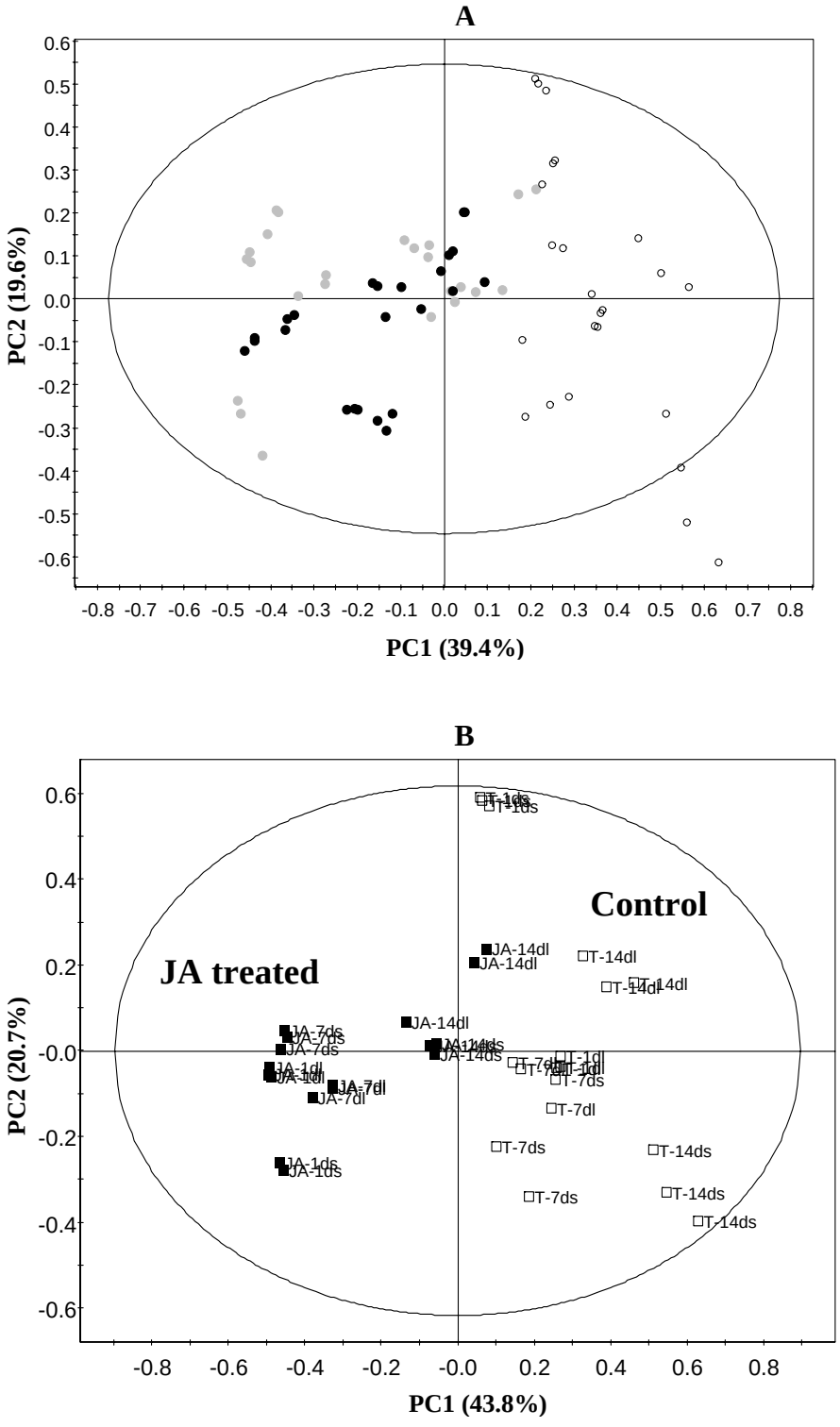
The significance of differences of NMR peak areas of some selected metabolites contributing in the discrimination of control and treated plants with JA and SA was assessed by t-test using Multi-Experiment Viewer (MEV) version 4.0 (Saeed *et al*., 2003). Also the significance of the differences between individual and total glucosinolates in treated plants compared to those of control was evaluated by t-test. Principal component analysis (PCA) was performed for the NMR data with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden).

7.3 Results and discussion

7.3.1 Metabolomic profiling of treated *Brassica rapa* with JA and SA by NMR spectroscopy

Principal component analysis (PCA) of the metabolomic data of control and treated plants with JA and SA was carried out. The score plot of PC1 vs. PC2 showed a clear separation between control and treated plants (**Figure 7.1A**). Although there is a clear discrimination between control and treated plants with JA and SA at each time point, overlapping of all time points makes it difficult to interpret the score plot of PCA. Thus, PCA was carried out for JA separately from SA treatment. The separated score plots of PCA of JA or SA treated plants showed a clear separation of treated plants from controls (**Figure 7.B, D**). JA treated plants were characterized by higher

levels of secondary metabolites such as phenylpropanoids (sinapoyl-, coumaroyl-, feruloyl- and 5-hydroxyferuloyl malate), flavonoids (kaempferol and quercetin), aliphatic glucosinolates (progoitrin) and indole glucosinolate (neoglucobrassicin), IAA, steroids and primary metabolites such as amino acids (alanine, glutamic acid and GABA) and fatty acids (**Figure 7.1C**).



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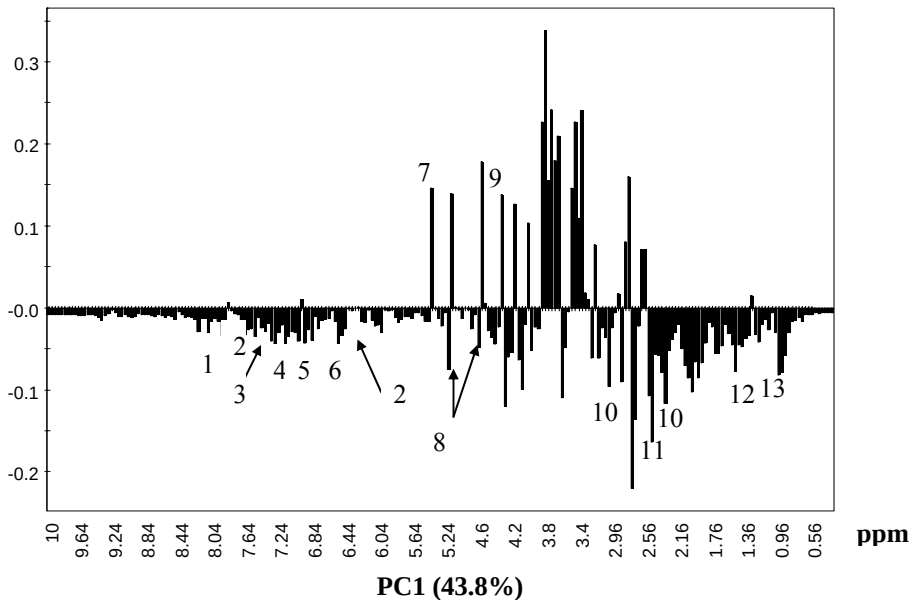
0.3
0.2
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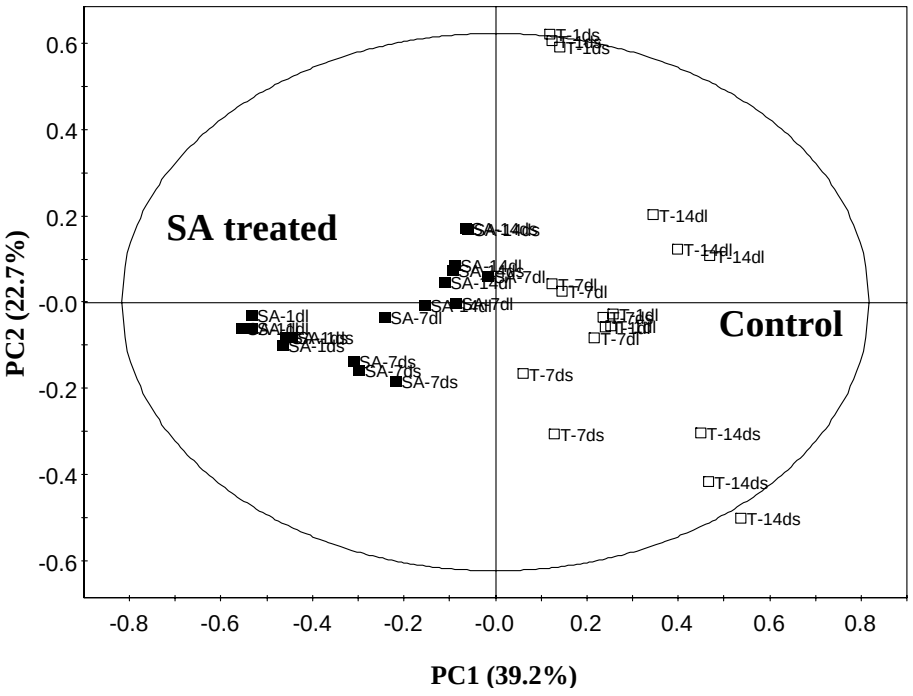
ppm
PC1 (43.8%)

D


SA treated
Control
PC2 (22.7%)
PC1 (39.2%)
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8 -0.6 -0.4 -0.2 -0.0 0.2 0.4 0.6 0.8
T-1ds
T-14dl
T-14dl
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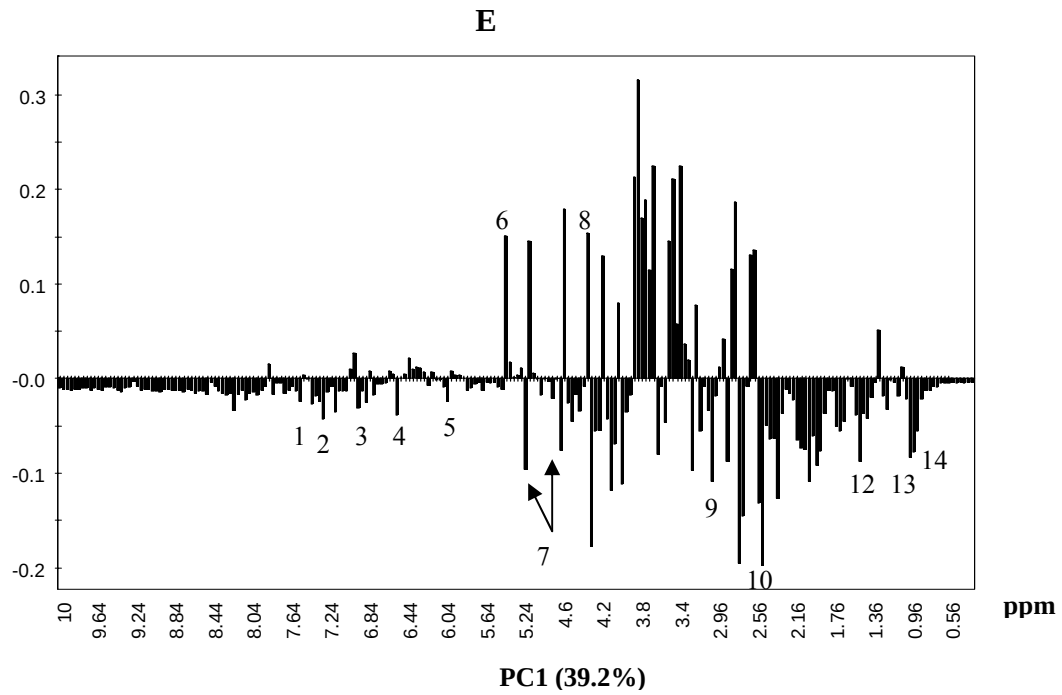

Figure 7.1. The score scatter plot of PC1 vs. PC2 of control and treated plants with JA and SA (**A**), Control plants (○), JA treated plants (●) and SA treated plants (●), the score plot of PCA (PC 1 vs. PC2) (**B**) and loading column plot of PC 1 (**C**) of *Brassica rapa* treated with JA. Score plot of PCA (PC1 vs. PC2) (**D**) and loading column plot of PC 1 (**E**) of *Brassica rapa* treated with SA. Assigned signals of **C**, 1: kaempferol, 2: *trans*-phenylpropanoids, 3: neoglucobrassicin, 4: IAA, 5: quercetin, 6: fumaric acid, 7: sucrose, 8: glucose, 9: malic acid, 10: GABA, 11: glutamic acid, 12: alanine, 13: fatty acids. Assigned signals of **E**, 1: neoglucobrassicin, 2: IAA, 3: coumaroyl malate, 4: fumaric acid, 5: progoitrin, 6: sucrose, 7: glucose, 8: malic acid, 9: GABA, 10: succinic acid, 11: glutamic acid, 12: alanine, 13: fatty acids, 14: steroids.

SA treated plants were characterized by higher levels of IAA, steroids, glucosinolates (progoitrin and neoglucobrassicin) and primary metabolites such as amino acids (alanine, glutamic acid and GABA), fumaric acid, succinic acid and fatty acids (**Figure 7.1E**). JA and SA treated plants showed significant decrease in the content of sucrose, choline and malic acid. The metabolites responsible for discrimination between controls and treated plants were tested by t-test using Multi-Experiment Viewer (MEV) (Saeed *et al*., 2003).

In the score plot of all time points used, treated plants with JA after 7d showed the clearest differences from controls. JA and SA effectively induced glucosinolate levels in *Brassica* species after 7d (Bodnaryk, 1994; Kiddle *et al*., 1994). So we subjected NMR data 7d after JA or SA treatment to multivariate data analysis. Score and loading plots revealed that JA treated plants showed higher concentrations of secondary metabolites such as phenylpropanoids (sinapoyl-, coumaroyl- and 5-hydroxyferuloyl malate), flavonoids (kaempferol and quercetin), IAA, steroids, progoitrin and neoglucobrassicin and higher levels of primary metabolites such as amino acids (alanine, threonine, valine and GABA), adenine, fumaric acid, succinic

acid and fatty acids (**Figure 7.2A and Figure 7.3**). SA treated plants were characterized by higher concentrations of secondary metabolites (kaempferol, steroids, progoitrin and neoglucobrassicin), primary metabolites including amino acids (alanine, threonine and GABA), adenine and fatty acids (**Figure 7.2B, Figure 7.3**). Where JA treated plants showed significant decrease in the content of sucrose, choline and malic acid, SA treated plants also showed on top of that a significant decrease in feruloyl- and caffeoyl malate.

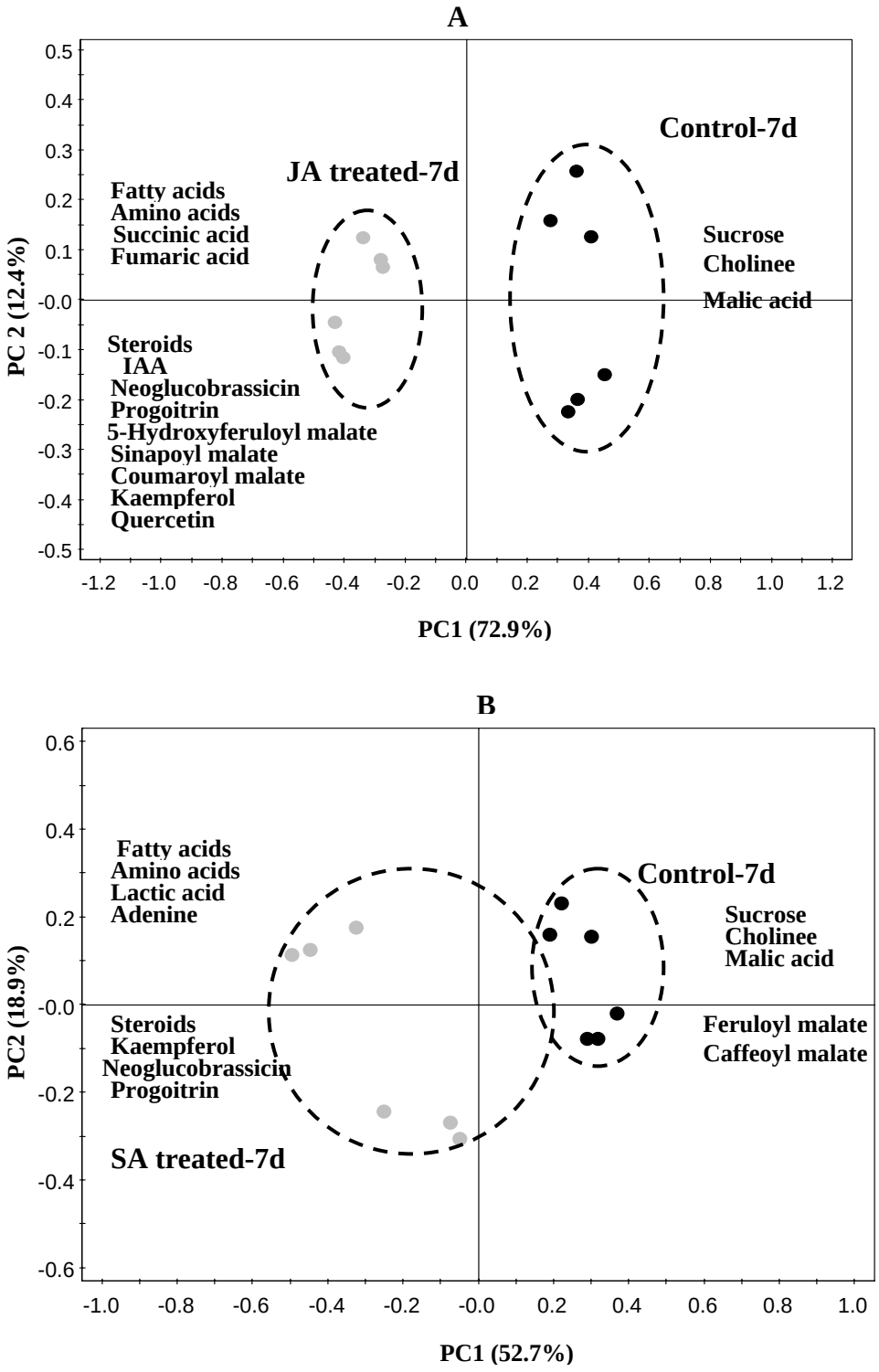

Figure 7.2. The score plot of PCA (PC 1 vs. PC2) (**A**) of *Brassica rapa* treated with JA after 7d. Score plot of PCA (PC1 vs. PC2) (**B**) of *Brassica rapa* treated with SA after 7d.

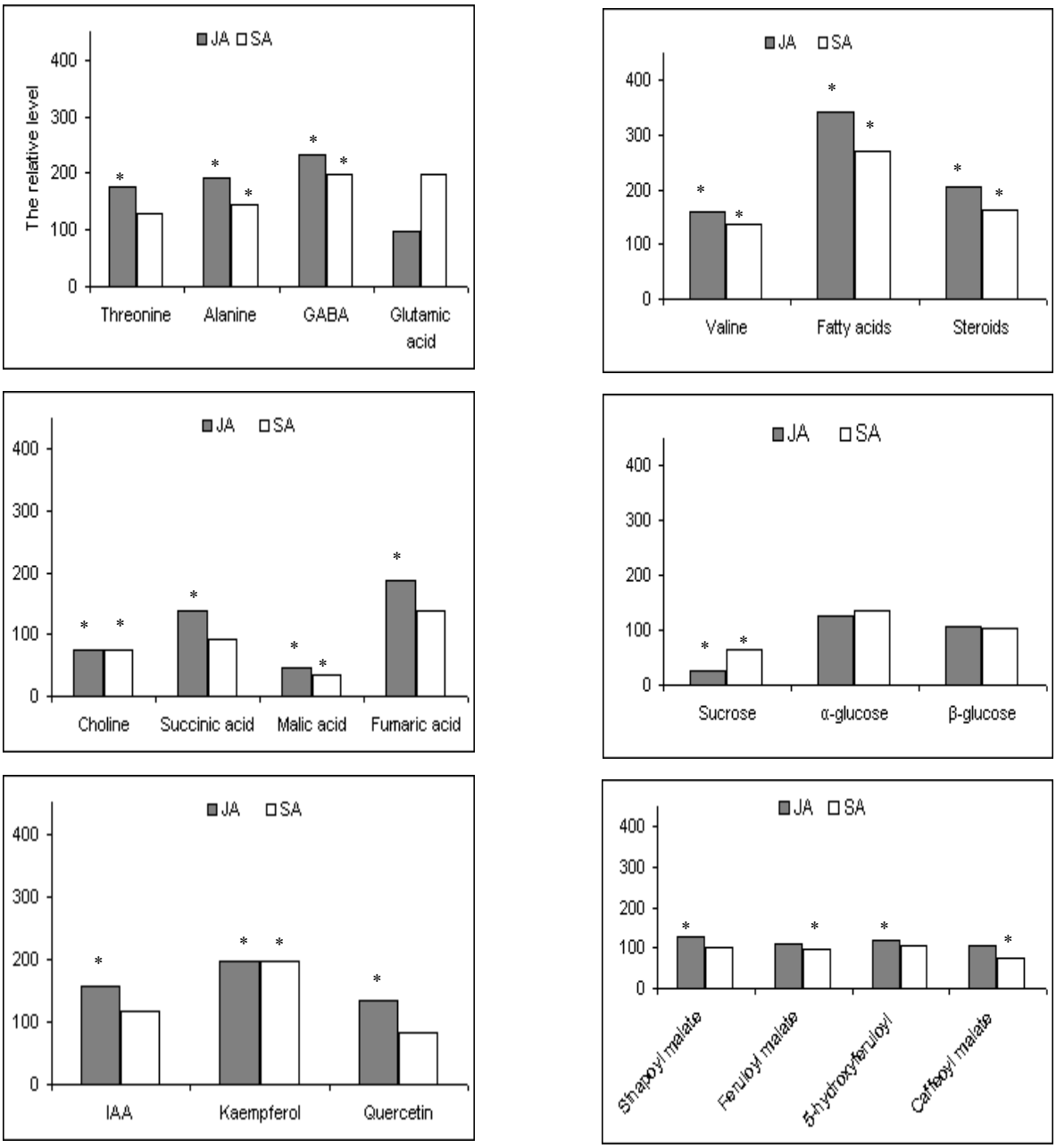

Figure 7.3. Relative levels compared to 100% of control of metabolites 7d after JA and SA treatment. *=significant at $p < 0.05$.

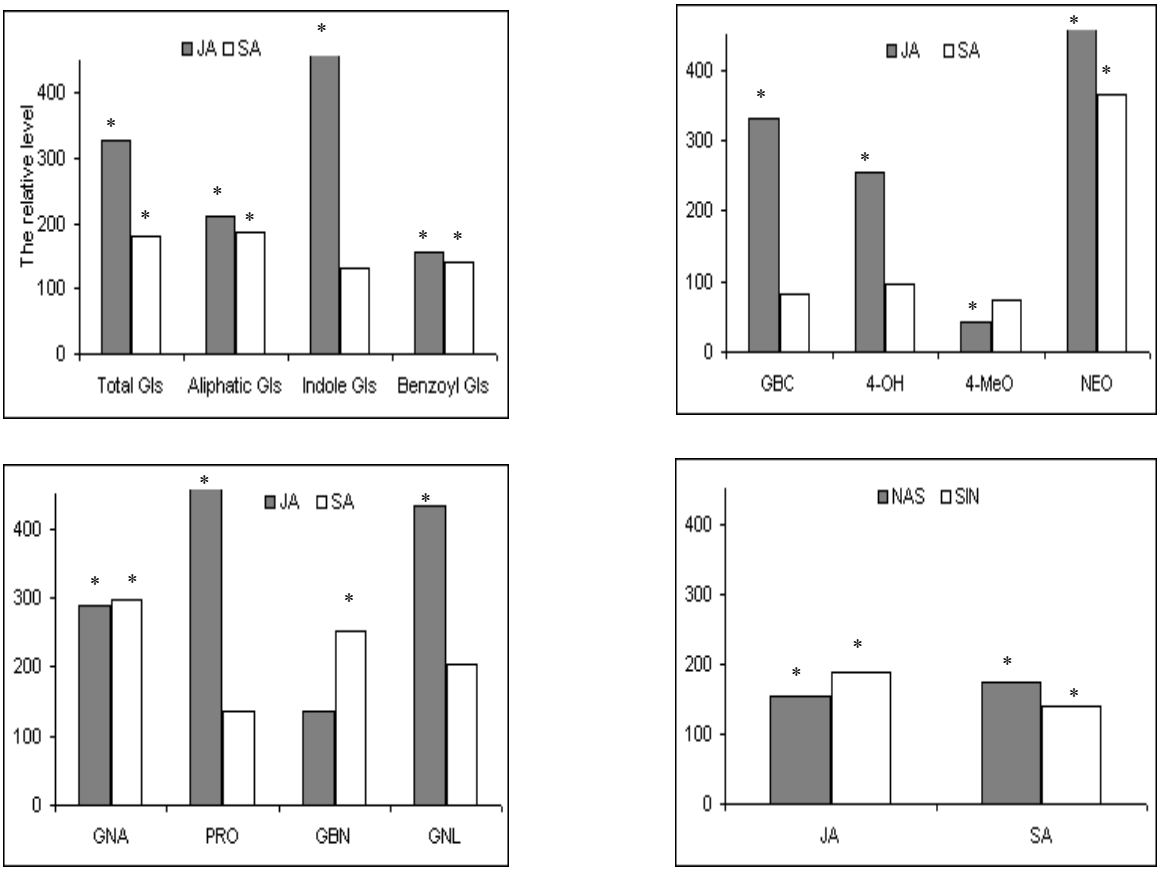

Figure 7.4. Relative levels compared to 100% of control of glucosinolates 7d after JA and SA treatment. GBC: glucobrassicin, 4-OH: 4-hydroxy-glucobrassicin, 4-Me: 4-methoxy-glucobrassicin, NEO: Neoglucobrassicin, GNA: gluconapin, PRO: Progoitrin, GBN: glucobrassicanapin, GNL: gluconapoleiferin, NAS: gluconasturtiin, SIN: sinalbin. *=significant at $p < 0.05$.

7.3.2 Glucosinolates profiling of *Brassica rapa* treated with JA and SA by HPLC

To further study the changes in glucosinolate levels, a more targeted HPLC analysis of glucosinolates was performed. **Figure 7.4** shows the relative levels of glucosinolates comparing to 100% of controls after 7d from JA and SA treatment.

Total, aliphatic, indole and benzoyl glucosinolates increased significantly after JA treatment, where total, aliphatic and benzoyl glucosinolates increased significantly after SA treatment. Some of the aliphatic glucosinolates (gluconapin, progoitrin and gluconapoleiferin), indole glucosinolates (glucobrassicin, neoglucobrassicin and 4-hydroxy-glucobrassicin) and benzoyl glucosinolates (gluconasturtiin and sinalbin) were significantly increased in JA treated plants, whereas two aliphatic glucosinolates (gluconapin and glucobrassicanapin), one indole glucosinolates (neoglucobrassicin) and benzoyl glucosinolates (gluconasturtiin and sinalbin) were significantly increased in SA treated plants (**Figure 7.4**).

The distribution of metabolites in different time points after JA and SA treatments was studied. The results indicated that in JA treated plants, phenylpropanoids, flavonoids aliphatic, indole, benzoyl glucosinolates and IAA significantly increased after 7d from treatment. Where in SA treated plants, phenylpropanoids, flavonoids and IAA showed lower levels than controls after 1 and 7d from treatment. SA treated plants showed higher levels of benzoyl and indole glucosinolates after 1d from treatment (**Figure 7.5**).

Unfortunately there are few publications related to the effect of signal molecules on the whole metabolome of *B. rapa* especially concerning the effect of SA. To our knowledge only Liang *et al*. (2006b) studied the metabolome alteration of *B. rapa* after MJ treatment. The authors concluded that the MJ treated plants showed a decrease of carbohydrates levels, whereas phenylpropanoids, IAA and indole glucosinolates increased in treated plants. Hendrawati *et al*. (2006) reported that in MJ treated *Arabidopsis* the amounts of alanine, threonine, valine, flavonoids, aliphatic glucosinolate (sinigrin), sinapoyl malate and tryptophan increased, whereas the concentration of malic acid, carbohydrates and feruloyl malate decreased. This is consistent with our results which showed an increase not only in phenylpropanoids (sinapoyl-, coumaroyl- and 5-hydroxy-feruloyl malate), flavonoids (kaempferol and quercetin), steroids, IAA, aliphatic and indole glucosinolate content but also an increase in a series of other compounds including amino acids, succinic, fumaric acids and fatty acids (**Figures 7.3 and 7.4**).

Biotic stresses induce specific defense responses in plants and affect the secondary metabolism (Tebayashi *et al*., 2000). Particularly phenolics are affected as e.g. Barley leaves treated with MJ accumulated a hydroxylcinnamic acid amide, *p*-coumaroylagmatine (Lee *et al*., 1997) and MJ treated hairy root cultures of *Daucus carota* showed an enhanced accumulation of *p*-hydroxy benzoic acid (*p*-HBA) and of the total phenolic content (Sircar and Mitra, 2008). Plants treated with JA showed pronounced accumulation of glucosinolates after 7d from treatment (**Figure 7.2A and 7.4**). Aliphatic glucosinolates dominated by progoitrin and gluconapoleiferin and indole glucosinolates dominated by glucobrassicin, 4-hydroxy-glucobrassicin and neoglucobrassicin and benzoyl glucosinolates (glucoasturtiin and sinalbin) accumulated in high levels after JA treatment. MJ sprayed *B. napus* accumulated progoitrin, glucobrassicanapin, glucobrassicin and neoglucobrassicin together with benzoyl glucosinolate (gluconasturtiin) after 7d from treatment (Doughty *et al*.,

1995). Loivamäki *et al*. (2004) showed enhancement of gluconapoleiferin, glucobrassicin and 4-hydroxy-glucobrassicin in *B. rapa* subsp. *oleifera* cv. Tuli treated with MJ. 4-Hydroxy-glucobrassicin increased significantly after 7d from treatment in the second cultivar (Valo) (Loivamäki *et al.*, 2004). MJ was able to trigger indole glucosinolates in *Arabidopsis* (Brader *et al*., 2001). The main compounds that enhanced after treatment of *Brassica* with MJ and JA were indole glucosinolates (glucobrassicin, 4-hydroxy-glucobrassicin and neoglucobrassicin) (Bodnaryk, 1994; Doughty *et al*., 1995). Treatment of different species of *Brassica* with JA or MJ enhanced the accumulation of specific indole glucosinolates in cotyledons and leaves: 4-hydroxy-glucobrassicin was induced in *B. rapa*, neoglucobrassicin in *B. napus* and both glucobrassicin and neoglucobrassicin were induced in *B. juncea* (Bodnaryk, 1994). Elicitor from the plant pathogen *Erwinia carotovora* triggered induction of the tryptophan biosynthesis pathway leading to increased levels of glucobrassicin in *Arabidopsis* (Brader *et al*., 2001). It seems that the effect of JA on glucosinolate contents is not similar to that of infection by pathogenic fungi where we can observe enhancement of individual glucosinolates of different classes (aliphatic, indole and benzoyl) after treatment and enhancement of benzoyl glucosinolates and 4-methoxy-glucobrassicin after fungal infection (**Chapter 6**). The reduction of glucobrassicanapin and gluconapin after 7d from JA treatment in our experiment might be attributed to the conversion of glucobrassicanapin and gluconapin to gluconapoleiferin and progoitrin, respectively through hydroxylation process which showed a significant increase in progoitrin and gluconapoleiferin after JA treatment (**Figure 7.4**).

SA treated plants showed significant decrease of some phenylpropanoids such as feruloyl- and caffeoyl malate, where other phenylpropanoids did not significantly change. Flavonoids (kaempferol), steroids, glucosinolates, primary metabolites including amino acids and fatty acids increased significantly with decreasing of sucrose, malic acid and choline after 7d from treatment (**Figures 7.2B, 7.3 and 7.4**).

SA treated *B. oleracea* showed higher concentrations of anthocyanins (Cole, 1996). The decrease of phenylpropanoids after 1 and 7d from SA is different with previous reports which showed an accumulation of phenolics directly after treatment of grape berries with SA. Particularly caffeic-, *p*-coumaric-, ferulic- and sinapic acids increased significantly after post-harvest application of SA with a maximum accumulation appearing 1d after treatment (Chen *et al*., 2006).

Also in *Citrus sinenisis* fruits, SA enhanced the production of the total phenolics and total flavonoids (Huang *et al*., 2008). Apparently these pathways are regulated differently in different species. The increase of IAA, indole and benzoyl glucosinolates after 1d from SA treatment may indicate the channeling precursors through biosynthesis of glucosinolates and IAA on the expense of flavonoids and phenylpropanoids (**Figure 7.5**).

Regarding to glucosinolate profiling, gluconapin, glucobrassicanapin, neoglucobrassicin, gluconasturtiin and sinalbin accumulated in high levels after SA treatment (**Figure 7. 4**). SA treated *Brassica campestris* ssp. *pekinensis* showed enhanced accumulation of indole glucosinolates dominated by neoglucobrassicin and 4-methoxy-glucobrassicin in treated roots and also in leaves together with gluconapin and gluconasturtiin (Ludwig-Muller *et al*., 1997). Glucosinolates showed different patterns of accumulation in different *Brassica* species after treatment with JA, MJ or SA. Aliphatic glucosinolates are synthesized from methionine and a small number of genes regulate side-chain elongation and side-chain modification giving different aliphatic glucosinolates. The pathways of the three classes of glucosinolates are shown in **Figure 7.6**. The qualitative and quantitative differences observed among aliphatic glucosinolates composition may be due to the allelic variation in a few genes encoding key regulatory enzymes at key points in the glucosinolate pathway (Halkier and Du, 1997). The hydroxylation of gluconapin and glucobrassicanapin to produce progoitrin and gluconapoleiferin is activated after JA treatment but not after SA treatment.

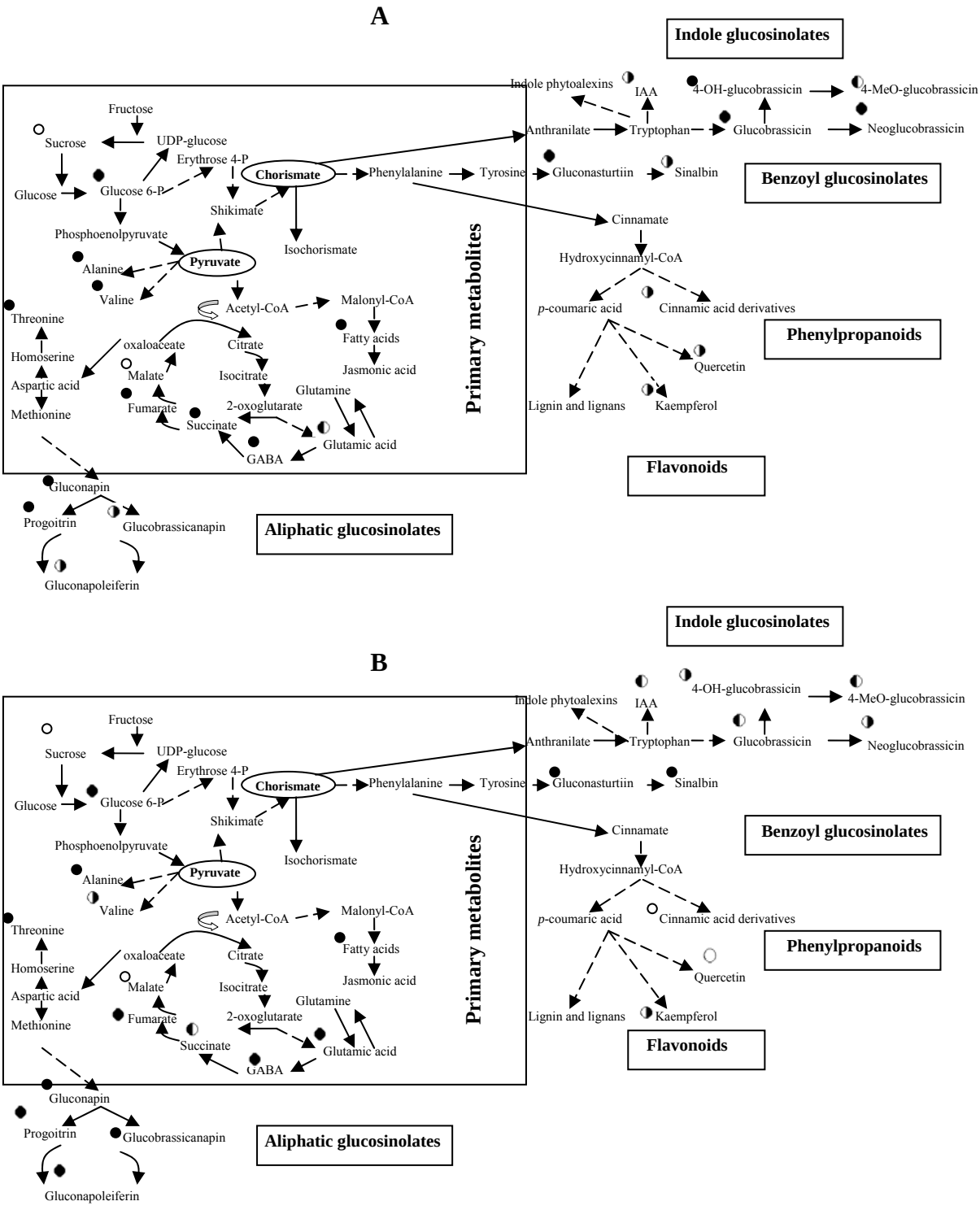

Figure 7.5. Metabolite linkage map between primary and secondary metabolism in *Brassica rapa* treated with jasmonic acid (**A**) and salicylic acid (**B**). Dashed lines, multi-steps reactions and solid lines, one-step reactions. ●, increased after 1 and 7d; ○, decreased after 1 and 7d; ◐, increased after 1d and decreased after 7d; ◑, decreased after 1d and increased after 7d.

JA treated plants showed higher concentrations of progoitrin and gluconapoleiferin and SA treated plants showed higher concentrations of gluconapin and glucobrassicanapin. Neoglucobrassicin and 4-hydroxy-glucobrassicin increased significantly after JA treatment and neoglucobrassicin after SA treatment. This may be attributed to hydroxylation and *N*-*O*-methylation of part of glucobrassicin after JA and only *N*-*O*-methylation after SA treatment. This can be one factor that controls the qualitative and quantitative differences observed among the aliphatic and indole glucosinolates (**Figure 7.6**).

In our experiments the sucrose content, choline and free malic acid decreased after JA or SA treatment. The decrease of sucrose levels in treated plants with JA or SA may be a mechanism for reallocation of valuable nutrients to other un-treated plant parts for compensatory growth (Rostás *et al*., 2002). The conversion of sucrose by the increased activity of the invertase enzyme yielding fructose and glucose (André *et al*., 2005) and using the latter for biosynthesis of secondary metabolites such as glucosinolates and flavonoids can be another explanation for the reduction of sucrose amounts in treated plants.

Our results provide conclusive evidence that treatment of *B. rapa* with JA and SA increases the accumulation of antifungal secondary metabolites including glucosinolates, phenylpropanoids, IAA and flavonoids. The accumulation of some primary metabolites such as amino and organic acids after JA and SA treatment might be connected with the demand for these compounds as substrates for secondary metabolite pathways connected with the plant defense. Increase of the amino acids GABA, glutamic acid, alanine, threonine and valine after treatment with JA and SA raises a question about their role in plant resistance, for example significant increases have been reported for *Brassica rapa* exposed to abiotic stress such as heavy metals application (Jahangir *et al*., 2008b) and to biotic stress such as infection with pathogenic bacteria (Jahangir *et al*., 2008a), infection with *Agrobacterium tumefaciens* (Simoh, 2008) and with pathogenic fungi (**Chapter 6**).

Considering all the results obtained in this study, we propose the metabolites linkage as shown in **Figure 7.5**. SA treated plants are characterized by low levels of phenylpropanoids, flavonoids (quercetin) and IAA compared to control plants and by higher levels of kaempferol, indole glucosinolates (neoglucobrassicin) and benzoyl glucosinolates (gluconasturtiin and sinalbin).

This points to an important role for the regulation of phenyalanine and tyrosine through biosynthesis leading to increased levels of kaempferol and benzoyl glucosinolates and regulation of tryptophan through biosynthesis leading to increased neoglucobrassicin. JA treated plants are characterized by higher levels of phenylpropanoids, flavonoids and indole glucosinolates as well as IAA which indicates the regulation of phenyalanine and tyrosine leading to increased levels of phenylpropanoids, flavonoids and benzoyl glucosinolates and regulation of tryptophan leading to increased levels of IAA and indole glucosinolates (glucobrassicin, 4-hydroxy-glucobrassicin and neoglucobrassicin).

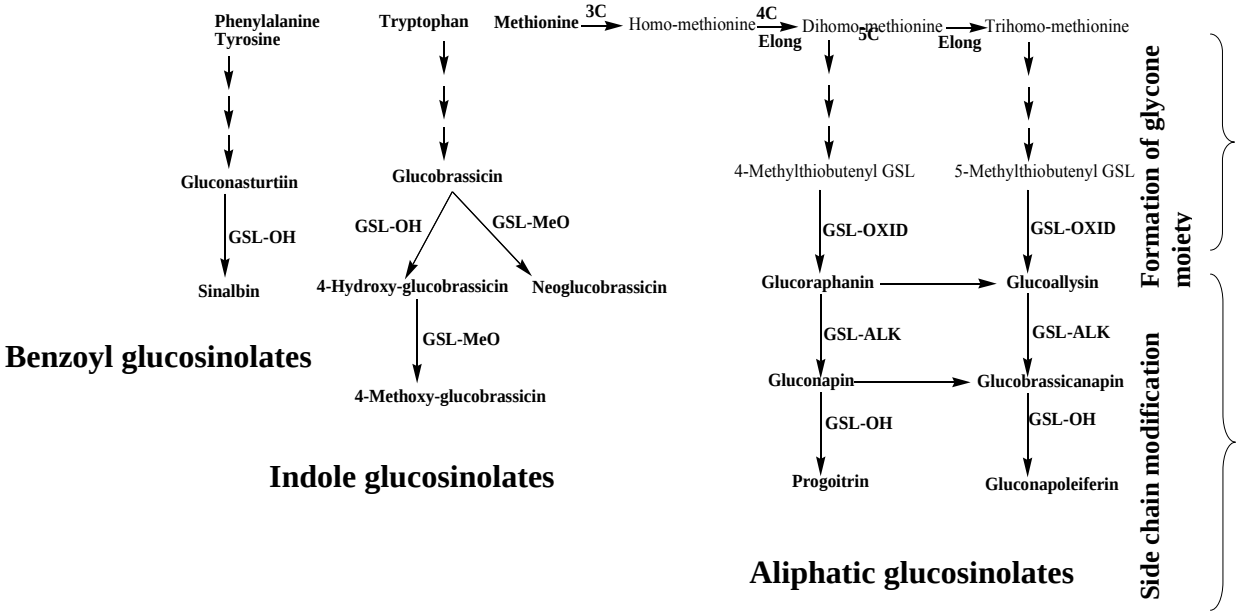

Figure 7.6. Metabolic networks of the biosynthesis of different classes of glucosinolates including the major genes and enzymes controlling the biosynthetic pathway (modified after Halkier and Lu, 1997; Padilla *et al*., 2007).

7.4 Conclusion

Nuclear magnetic resonance spectroscopy (NMR)-based metabolomics combined with multivariate data analysis (PCA) is a powerful tool to study the effect of JA and SA on the *B. rapa* metabolome. Targeted HPLC analysis supported the data obtained by NMR and is an important complementary tool since it gives more information on the individual metabolites such as in this case glucosinolates, often present in low concentrations. The effects of SA and JA in part overlap but particularly for the phenylpropanoids and in the indole glucosinolate biosynthetic networks there are clear differences. This makes this system an interesting model to study reallocation of carbon fluxes under different forms of stress.