Metabolic changes in Arabidopsis thaliana plants overexperssing chalcone synthase

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Chapter 8

Effect of benzothiadiazole on the metabolome of *Arabidopsis thaliana*

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Abstract

Benzothiadiazole (BTH) is a functional analogue of the plant endogenous hormone-like compound, salicylic acid (SA), which is required for the induction of plant defense genes leading to systemic acquired resistance (SAR). Previous molecular and genetic studies have suggested that BTH itself might potentiate SAR resulting in the induction of several pathogenesis-related (PR) genes. However, the changes in the metabolome, which occur as a result of BTH-treatment, remain unclear. In this study, metabolic alterations in BTH-treated *Arabidopsis thaliana* were investigated using nuclear magnetic resonance (NMR) spectroscopy followed by multivariate data analyses such as principal component analysis (PCA) and partial least square - discriminant analysis (PLS-DA). Both PCA and PLS-DA show that increase of glucose, glutamine, inositol, malic acid, sucrose, and threonine as well as BTH and its degraded metabolites contribute to the clear discrimination of the metabolome of BTH-treated *Arabidopsis* from control plants. However, the levels of phenolic metabolites which have generally been observed to be induced by other signaling molecules were significantly reduced in BTH-treated *Arabidopsis.* In addition to these changes due to BTH-treatment, it was

also found that the ethanol used as a solvent in this treatment may *per se* act as an inducer of the accumulation of a flavonoid.

Keywords: *Arabidopsis thaliana*, Benzothiadiazole, Metabolomics, Nuclear magnetic resonance spectroscopy, Systemic acquired resistance, Principal component analysis, Partial least square - discriminant analysis

8.1. Introduction

Plants interact constantly with the environment, e.g. other organisms, soil, climate, water conditions, or exogenous chemicals. When challenged, plants can switch on their defense mechanism in general, as a response to many stimuli, or specifically, responding to a certain stimulus. Among the plant defense mechanisms, systemic acquired resistance (SAR) is a whole-plant resistance response that follows an earlier local exposure to a pathogen. Fungal, bacterial, or viral pathogenic infections induce SAR, involving different biochemical pathways that produce salicylic acid (SA) among others, transduction signals of pathogenesis-related (PR) proteins, and/or phytoalexins [Bol *et al.,* 1990; Dixon, 1986; Felton *et al.,* 1999; Kombrink *et al.,* 1997; , Métraux *et al.,* 1990]. SAR can also be induced by exposing the plant to virulent, nonpathogenic microbes, or to chemicals such as SA, 2,6-dichloro-isonicotinic acid (INA) or benzo(1,2,3)thiadiazole-7-carbothioic acid-*S*- methyl ester (BTH) [Kuć, 1982; Ryals *et al.,* 1996; Sticher *et al.,* 1997].

The phenomenon of pathogen-induced SAR has been recognized as a plant response to pathogen infection for almost 100 years and has therefore been extensively studied in many plants at a genetic and proteomic level [Ryals *et al.,* 1996]. SAR is associated with the induction of gene expression of defensive factors such as PR proteins, and this activation requires the production of endogenous SA [Métraux *et al.,* 1990]. Several PR proteins including PR-1, PR-2 (β-1,3-glucanases), PR-3 (chitinases), PR-4, and PR-5 (osmotin) were found positively correlated with the onset of SAR although with an expression level of marker genes for SAR that varied between different species [Kessmann *et al.,* 1994;, Vleeshouwers *et al.,* 2000]. In *Arabidopsis thaliana,* the mRNAs for PR-1, PR-2, and PR-5 accumulated in a coordinated manner in tissues that became resistant after pathogen infection [Uknes *et al.,* 1993]. Most PR proteins were found to accumulate in the extracellular space or in the vacuole. The extracellular PR proteins are thought to be directly in contact with the pathogen penetrating the tissue and vacuole PR proteins are probably involved in the following defense reaction after decompartmentalization [Sticher *et al.,* 1997]. Different roles have been attributed to PR proteins, such as antimicrobial or antifungal activities *in vitro* activities [Liu *et al,* 1994; Mauch *et al.,* 1988] or the capacity of releasing elicitors [Kurosaki *et al,* 1986]. However, the exact role of PR proteins in SAR still remains unclear.

Benzo(1,2,3)-thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH, Fig. 1) is a potent SAR activator which provides protection in natural conditions against a broad spectrum of diseases affecting a variety of crops [Friedrich *et al.,* 1996; Görlach *et al.,* 1996; Lawton *et al.,* 1996]. Although BTH is a strong SAR inducer which causes the expression of the same set of SAR genes as those induced by SA, it does not require accumulation of SA but may act downstream of SA [Friedrich *et al.,* 1996; Görlach *et al.,* 1996;, Kuć 1982]. BTH induces SAR in tobacco [Friedrich *et al.,* 1996], wheat [Görlach *et al.,* 1996] and in *Arabidopsis* [Lawton *et al.,* 1996]. In the latter, BTH was found to directly activate PR-1 and to prime the plants for potential phenylalanine ammonia-lyase (PAL) expression in response to the infection by phytopathogenic *Pseudomonas syringae* p.v. tomato (Pst) [Lawton *et al.,* 1996]. It is also an excellent elicitor for the SA-activated defensive pathways in cotton, inducing remarkable levels of activity of PR proteins both locally and systemically [Inbar *et al.,* 2001]. At the metabolome level, BTH proved unable to induce any specific metabolites itself, even though there was a significant induction of PR genes and proteins [Katz *et al.,* 1998]. A remarkable change at this level was detected only after elicitation [Katz *et al.,* 1998], implying that BTH can only potentiate plants following elicitation or infection by induction of PR protein genes. In contrast with these findings, interesting results were recently reported about the metabolic variation of grapevine following BTH-treatment [Iriti *et al.,* 2004]. In this case, total polyphenols such as stilbenoids, flavonoids, anthocyanidins, and proanthocyanidins increased notably in the plants after BTH-treatment. This report awakened our interest in studying possible metabolomic changes in BTH-treated *Arabidopsis* since there is very scarce information on this aspect as compared to the knowledge of transcriptomic and proteomic levels of BTH-treated plants.

Changes at a transcriptomic and proteomic level should necessarily be reflected in the metabolome, since metabolites are the final amplified product of gene and protein expression. In recent years, metabolomics studies have received increasing attention, as a means of acquiring a better insight into the complete biological process, combining this information with that obtained through genomics, transcriptomics and proteomics [Hirai *et al.,* 2004; Kolbe *et al.,* 2006; Oksman and Saito, 2005]. Technological advances in analytical chemistry and instrumentation have accelerated the development

of diverse tools for metabolomics, particularly the information technology and mathematics needed to deal with the handling of large datasets, which have played a major role in developing the full potential of these analytical methods. Among these, it is generally accepted that NMR is the optimal tool for macroscopic metabolomics [Choi *et al.,* 2006; Verpoorte *et al.,* 2007]. This is especially the case when ^{1}H-NMR spectroscopy is applied to metabolomics since a diverse group of metabolites including amino acids, carbohydrates, lipids, phenolics, and terpenoids can be detected simultaneously [Choi *et al.,* 2004; Hendrawati *et al.,* 2006; Liang *et al.,* 2006]. It is also an easier and more robust method for acquiring quantitative raw data when compared to other methods. These positive features of NMR have led many researchers to use NMR as the first choice of plant metabolomics.

In this study NMR spectroscopy and multivariate data analysis including principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were applied to the analysis of metabolic changes in *Arabidopsis thaliana* treated by the SAR inducing chemical, BTH. Based on the results, the induction or suppression of diverse metabolites following BTH treatment in *Arabidopsis* as compared to control plants was investigated. The information thus obtained is expected not only to provide knowledge on metabolic characteristics but also to advance the understanding of the molecular basis of systemic acquired resistance in plants.

8.2. Methods

8.2.1. Reagents

Analytical grade CH_3OH and $CHCl_3$ were purchased from Merck Biosolve Ltd. (Valkenswaard, The Netherlands). CH_3OH-d_4 and D_2O (99.0%) were obtained from Cambridge Isotope Laboratories Inc (Miami, FL, USA) and NaOD was purchased from Cortec (Paris, France). Potassium dihydrogen phosphate (KH_2PO_4) and trimethylsilane propionic acid sodium salt (TMSP) were bought from Merck (Darmstadt, Germany). Benzo(1,2,3)-thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH) was obtained form Novartis (Basel, Switzerland).

8.2.2. Growing Arabidopsis

Seeds of *Arabidopsis thaliana* ecotype Col-0 were generously donated by Dr. J. Memelink (Institute of Biology, Leiden University, Leiden, The Netherlands). Seeds were sown in soil and kept at 4 °C for 4 days. The *Arabidopsis* plants were grown under identical long-day controlled environment conditions in trays, 25 °C, and 16 h light/8 h dark. Four groups of *Arabidopsis* were prepared including BTH (in 80% EtOH)-treated, 80% EtOH-treated, and two control groups without any treatment which were grown in different locations under the same growing conditions. Four replicates were used for each time point of the groups. BTH or EtOH treatment was performed after 6 weeks growth.

8.2.3. BTH treatment

Treatments were carried out following the method of Lawton et al. with a BTH concentration of 300 µmol in 80% EtOH per plant [Lawton *et al.,* 1996]. Two groups of control *Arabidopsis* plants were prepared: one group was untreated and another was treated with the same amount of 80% EtOH as BTH treated ones. Plants were harvested at 4, 24, 48 and 96 h after treatment and frozen in liquid nitrogen. The whole aerial parts were homogenized and stored at - 80 °C until used.

8.2.4. RNA analysis

Total RNA was isolated using RNeasy plant mini kit (Qiagen, Hilden, Germany). The purified RNA was treated with DNase I using a DNA-free kit (Ambion, Austin, TX, USA) and the purity of RNA integrity was confirmed by running on a 1.5% (w/v) agarose gel. Total RNA (2 µg) was reverse transcribed in a 20 µl reaction using an oligo$(dT)_{18}$ primer and SuperScript™ II reverse transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The cDNA was diluted 20 times and 1 µl was used as template for a real-time PCR experiment. The primers used to amplify *PR-1* were 5'-GTAGGTGCTCTTGTTCTTCCC-3' and 5'-CACATAATTCCCACGAGGATC- 3'. The primers used to amplify *actin1* were 5'-ATGAAGCTCAATCCAAACGA-3' and 5'-CAGAGTCGAGCACAATACCG-3'.

Real-time PCR was performed on ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Samples were amplified in a 50 µl reaction containing 1×SYBR Green Master Mix (Eurogentec, Maastricht, The Netherlands) and

300 nM of each primer. The thermal profile consisted of 1 cycle at 95 °C for 3.5 min followed by 40 cycles at 95 °C for 0.5 min and at 60 °C for 1 min. Changes in gene expression as a relative fold difference between BTH treated samples and control ones were calculated using the comparative C_t ($2^{-\Delta\Delta Ct}$) method [Schmittgen *et al.,* 2000; Winer *et al.,* 1999]. *Actin1* was used as a reference gene to normalize for differences of the total RNA amount.

8.2.5. Extraction and fractionation of plant material

For the analysis of polar metabolites, plant material was pulverized in liquid nitrogen using a mortar and pestle and freeze dried. An aliquot of dried material (50 mg) was transferred to a microtube and 1.5 ml of 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.05% Trimethylsilane propionic acid sodium salt (TMSP, w/v) was added. The mixture was vortexed at room temperature for 1 min, sonicated for 20 min and centrifuged at 13,000 rpm at room temperature for 5 min. A volume of 800 µl of the supernatant was transferred to a 5 mm-NMR tube.

In the case of non-polar metabolite extraction, 20 mg of plant material, were submitted to the same method as that described for polar metabolites extraction and extracted with 4 ml of $CHCl_3$-CH_3OH (1:1, v/v) followed by vortexing for 30 seconds and sonication for 1 min. The sample was then centrifuged at 3,000 rpm for 20 min. The extracts were placed in a 25 ml-round bottom evaporation flask and dried in a rotary vacuum evaporator. The dried fractions were redissolved in 800 µl of CH_3OH-d_4.

8.2.6. NMR spectra measurements

^{1}H-NMR and J-resolved spectra were recorded at 25 ^{o}C on a 400 MHz Bruker AV-400 spectrometer. Each ^{1}H-NMR spectrum consisted of 256 scans requiring 17.3 min acquisition time with the following parameters: 0.15 Hz/point, pulse width (PW) = 45^o (3.3 µ sec), and relaxation delay (RD) = 2.0 sec and acquisition time (AQ) =2.0 sec. 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 zero-filled to 32 K points. The resulting spectra were manually phased and baseline corrected, and calibrated to TSP at 0.0 ppm, all using XWIN NMR (version 3.5, Bruker). Two dimensional presaturated J-

resolved ^{1}H-NMR spectra were acquired using 16 scans per 64 increments that were collected into 16 K data points, using spectral widths of 5208 Hz in F2 (chemical shift axis) and 50 Hz in F1 (spin-spin coupling constant axis). A 1.0 sec relaxation delay and 1.6 sec for acquisition time was employed, giving a total acquisition time of 55.39 min. Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine functions prior to double complex FT. ^{1}H-^{1}H-correlated spectroscopy (COSY) spectra were acquired with 1.0 sec relaxation delay, 4194 Hz spectral width in both dimensions. The heteronuclear multiple quantum coherence (HMQC) spectra were obtained with 1.4 sec relaxation delay, 4401 Hz spectral width in F2 and 20124 Hz in F1. The heteronuclear multiple bond correlation (HMBC) spectra were recorded with 1.4 sec for relaxation delay, 4251 Hz for F2 axis, 20124 for F1 axis. Qsine function was used both for HMQC and HMBC (SSB=2.0)

8.2.7. Multivariate data analysis

The ^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.8, Bruker Biospin). Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (δ 0.04) corresponding to the region of δ 0.30 – δ 8.48. The regions of δ 4.70 - δ 4.90 and δ 3.28 - δ 3.34 in 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) and δ 4.90 - δ 5.28 and δ 3.24 - δ 3.34 in CH_3OH-d_4 were excluded from the analysis because of the residual signal of solvent and water. Principal component analysis (PCA) and partial least square - discriminant analysis (PLS-DA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden). For scaling method Pareto and unit variance method were used for PCA and PLS-DA, respectively.

8.3. Results and Discussion

8.3.1. PR-1 expression in BTH-treated Arabidopsis

Prior to metabolic analysis the expression of the PR-1 gene was confirmed by qPCR since it is a specific marker of SAR in *Arabidopsis* [Ryals *et al.,* 1996]. The accumulation of the PR-1 gene in BTH treated samples was observed 4 h after treatment and increased after 24 h and 48 h, but decreased after 96 h. The ethanol used to dissolve BTH seemed to act as an inducer itself since in EtOH treated samples; the expression of PR-1 was also detected 4 h after treatment. However, after 24 h the level of PR-1

expression was similar again to that of the control plants (non treated plants, data not shown).

8.3.2. Principal component analysis of ^{1}H-NMR spectra of control, ethanol and BTH-treated Arabidopsis

No single extraction method makes it possible to isolate a complete metabolome, i.e. the whole profile of metabolites, owing to its huge diversity in terms of chemical properties. To overcome this problem, two different extraction solvents were used: CH_3OH-H_2O for polar, hydrophilic metabolites and $CHCl_3$-CH_3OH for the less polar ones. This last extract showed no discriminating metabolites between control, EtOH and BTH-treated *Arabidopsis*.

A previous ^{1}H-NMR metabolomic study of *Arabidopsis*, carried out on a CH_3OH-water extract showed a great amount of amino acids, carbohydrates, organic acids, and phenolics that were clearly detected in a single spectrum [Hendrawati *et al,* 2006]. Aside from these constitutive plant metabolites, one more point had to be considered in this study. When a non-volatile chemical is introduced into a plant, residues of the compound itself or eventually its degradation products will also be included in the analysis. The suitability of a selected analytical method for metabolomics is dependent on whether or not those exogenous chemicals will interfere. A typical ^{1}H-NMR spectrum of BTH-treated *Arabidopsis* in a mixture of CH_3OH-d_4 and KH_2PO_4 buffer (pH 6.0) (1:1) is shown in **Figure 8.1**. ^{1}H-NMR will allow the detection of very diverse compounds, without magnifying a certain group of metabolites. Thus, amino acids, carbohydrates, flavonoids, nitrogen-containing metabolites, and phenylpropanoids are observed. A limitation of one dimensional (1D)-NMR spectroscopy is the congestion of signals. It was solved using diverse two dimensional (2D)-NMR techniques. In particular, 2D-J-resolved spectra greatly facilitated the analysis of the phenolic region (**Figure 8.3**). For treated plants, as expected, the signals of residual BTH at δ 8.97 (H-6, d, 9.2 Hz), δ 8.60 (H-4, d, 7.2 Hz), and δ 7.95 (H-5, t, 8.0 Hz) were observed in the spectra (**Figure 8.1A**). Adjacent to these signals, similar types of resonances at δ 8.74, δ 8.28 and δ 7.84 were detected and identified as those of a product of hydrolysis of BTH (**Figure 8.2**). All known *Arabidopsis* metabolites were elucidated based on the chemical shifts and coupling constants observed which were confirmed by diverse 2D-NMR such

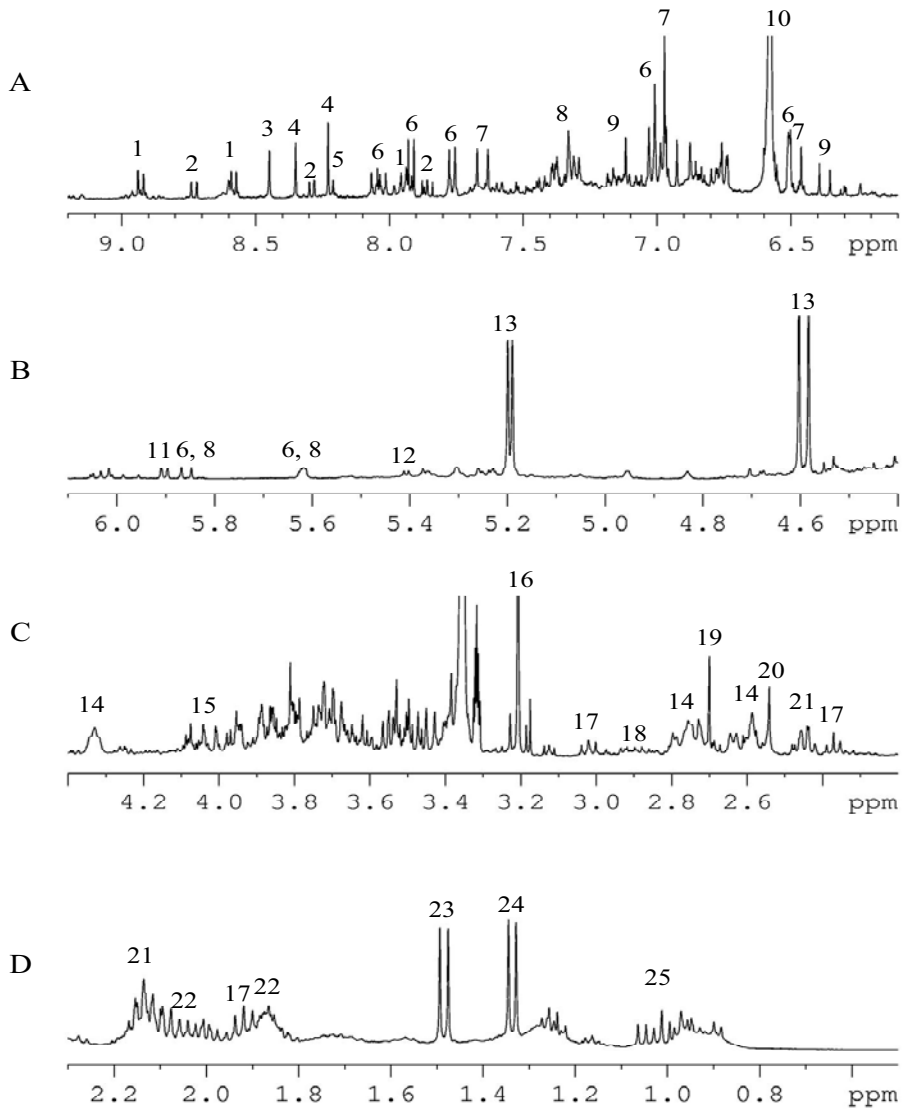


Figure 8.1. ^{1}H-NMR spectrum of *Arabidopsis thaliana* treated with BTH in 50% CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) in the range of δ 6.1 – δ 9.2 (A), δ 4.4 – δ 6.1 (B), δ 2.2 – δ 4.4 (C), δ 0.5 – δ 2.3 (D). 1; BTH, 2; hydrolyzed BTH, 3; formic acid, 4; adenosine, 5; adenine, 6; kaempferol glycosides, 7; sinapoyl malate, 8; quercetin-3-*O*-glycosides, 9; feruloyl malate, 10; fumaric acid, 11; cytosine, 12; sucrose, 13; *α* glucose, 14; malic acid, 15; inositol, 16; choline, 17; GABA, 18; asparagine, 19; diethylamine, 20; succinic acid, 21; glutamine, 22; proline, 23; alanine, 24; threonine, 25; valine

as J-resolved, correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra, and published data in our previous study [Hendrawati *et al.,* 2006].

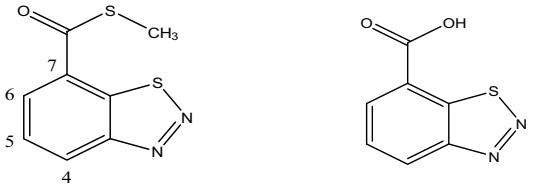


Figure 8.2. Chemical structures of BTH and its hydrolyzed metabolite.

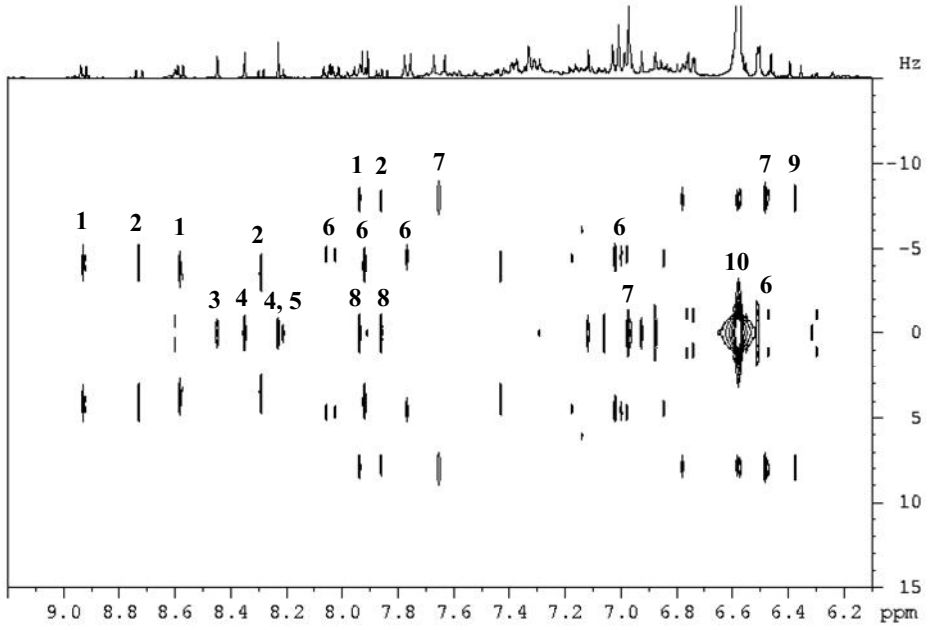


Figure 8.3. Two dimensional J-resolved spectrum of *Arabidopsis thaliana* treated with BTH in CH_3OH-d_4 in D_2O (KH_2PO_4 buffer, pH 6.0) in the range of δ 6.1 – δ 9.2). 1; BTH, 2; hydrolyzed BTH, 3; formic acid, 4; adenosine, 5; adenine, 6; kaempferol glycosides, 7; sinapoyl malate, 8; quercetin-3-*O*-glycosides, 9; feruloyl malate, 10; fumaric acid.

For a first overview of the metabolomic changes in BTH treated-*Arabidopsis,* principal component analysis (PCA) was applied to the ^{1}H-NMR data. Of the many multivariate data analysis method used in metabolomics, PCA is the most popular unsupervised grouping method for the reduction of the original data dimensions since the grouping or separation can be achieved simply based on maximum variation of samples without any biased information. In order to evaluate intact metabolic change, all the ^{1}H-NMR signals of BTH and its hydrolyzed metabolite were excluded for PCA.

Figure 8.4 shows the score plot of PCA based on ^{1}H-NMR spectra of the control sample, and 80% EtOH-treated and BTH-treated *Arabidopsis* (4, 24, 48 and 96 h after treatment). Three groups of control *Arabidopsis* plants were compared with BTH-treated ones. BTH treatment was carried out using a solution of BTH in 80% EtOH. It was therefore necessary to submit plants to a treatment with this solvent (as a control) in order to evaluate its activity. For non-treated controls, two batches of *Arabidopsis* were grown in different locations but under the same conditions, in order to assess possible biological variations. Metabolism is quite dynamic, being easily affected by many factors. Even under seemingly controlled conditions, level of metabolites might be affected by unpredictable external factors. However, while no separation was observed in the PCA score plot of the two control groups which had been grown in different locations (**Figure 8.4**), EtOH treated plants were slightly different from the two control groups. In accordance with reported PCR results, there is a possibility that the addition of EtOH may cause a weak induction of PR genes, which can result in a metabolic change. However, the EtOH-treated *Arabidopsis* are also separated from BTH-treated ones. As shown in Fig. 4, the BTH-treated plants are undoubtedly differentiated from others, especially 24 h after treatment. In general, change in metabolomic expression resulting from changes in the level of gene and protein levels can be expected to occur later. In fact most expression of genes related to BTH effect such as PR-1, PR-2, and PR-3 can be shown within 24 h [Wendehenne *et al.,* 1998] and metabolic alteration was clearly first detected after 24 h.

The signals responsible for the difference of BTH-treated *Arabidopsis* could be identified using the loading plot. The cluster of BTH-treated plants 24 h after treatment compared to the untreated controls or EtOH-treated samples, shows lower principal component (PC) 2 in the score plot of PCA. Using the loading plot, alanine, glucose,

glutamine, malic acid, and threonine were found positively related to the lower PC2 whereas aspartic acid, flavonoid glycosides, phenylpropanoids, and succinate were higher in the controls.

8.2.3. Partial least square-discriminant analysis of ^{1}H-NMR spectra of control, ethanol and BTH-treated Arabidopsis

While PCA is an excellent tool for data reduction and grouping multivariate data, it has limitations, which have to be considered. The separation by PCA is obtained only from maximum variations between samples as unsupervised multivariate data analysis is used. However, when information on some classification is available, it may be useful to apply a type of discriminant analysis, i.e. a supervised method in which grouping can be obtained by maximum covariance (e.g. metabolic difference corelated to classification). In this study, partial least square-discriminant analysis (PLS-DA) was employed using two classes; class 1 for control and EtOH-treated and class 2 for BTH-treated *Arabidopsis* plants. One of the advantages derived from the use of PLS-DA is that it concentrates on the effect of the selected factor (e.g. BTH or ETOH treatment) by reducing the influence of uninteresting factors such as the developmental stages of the plant.

The result obtained applying PLS-DA can be observed in **Figure 8.5**. Comparing this to the result of the PCA score plot (**Figure 8.4**), it is clear that a better separation between control (non-treated and EtOH-treated) and BTH-treated *Arabidopsis* plants is achieved. The separation in the score plot of PLS-DA could be obtained from the covariance between metabolites and treatments, disregarding the change due to developmental stages from 4 – 96 h or EtOH effect in which the metabolome were found to be greatly changed. According to PCA, the level of primary metabolites including alanine, glucose, glutamine, inositol, malic acid, sucrose, and threonine in BTH-treated samples were highly increased in a similar way. In previous studies, plants treated with signaling molecules such as analogues of jasmonic or salicylic acid, revealed a great variation in phenolics, which are known to play a role in plant defense [Görlach *et al.,* 1996; Kurosaki *et al.,* 1986; , Liang *et al.,* 2006]. However, BTH-treated plants exhibited a decrease of phenolics which could be explained considering that BTH may cause an accumulation of lignin by enhancing polar auxin transport [Besseau *et al.,* 2007; Katz *et*

al., 1998]. Additionally, numerous previous studies proved that BTH itself does not change metabolism but primes plant defense by activation of some specific genes, e.g. genes encoding peroxidases [Katz *et al.,* 1998] or by the formation of protective layers at the sites of attack [Benhamou and Belanger, 1998]. Resistance in terms of increased biosynthesis levels of defense compounds can be activated only by further elicitation after BTH treatment. For instance, phenylalanine ammonia-lyase (PAL), involved in the biosynthesis of many phenolics, did not increase in BTH-treated cultured parsley (*Petroselinum crispum* L.) but further elicitation greatly increased the mRNA level of PAL activity and coumarin secretion [Katz *et al.,* 1998].

In previous studies, trace amount of EtOH was found to alter the activity of certain enzymes [Li *et al.,* 2004]. For example, the activities of peroxidases and superoxidedismutase in cucumber roots highly increase by the addition of EtOH [Li *et al.,* 2004]. In the case *Ilex paraguariensis cell suspension* culture EtOH is glycosylated. However, the effect of EtOH on plant metabolims is still unclear. In our qPCR experiment, ethanol treated *Arabidopsis* expressed PR-1 at 4 h after treatment. Also, the metabolic change in EtOH-treated *Arabidopsis* was detected in the PCA score plot (**Figure 8.4**). In order to investigate the effect of EtOH on the metabolome of *Arabidopsis*, PLS-DA using two classes such as control and EtOH-treated samples was performed (**Figure 8.6**). When *Arabidopsis* was treated with 80% EtOH, a kaempferol glycoside as well as alanine, GABA, glucose, proline, and threonine, were unexpectedly induced. Two flavonoids, kaempferol- 3-*O*-D-glucopyranoside-7-*O*-L-rhamnopyranoside and kaempferol -3,7-*O*-L-dirhamnopyranoside, are the two major flavonoids reported in *Arabidopsis* [Hendrawati *et al.,* 2006]. Interestingly, only kaempferol 3-*O*-D-glucopyranoside-7-*O*-L-rhamnopyranoside was affected by the EtOH-treatment. However, this flavonoid was not found as a discriminating metabolite in BTH-treated plants although BTH was used in EtOH solution. It might be because BTH attenuate the effects of ethanol on the accumulation of the flavonoid in BTH-treated plants.

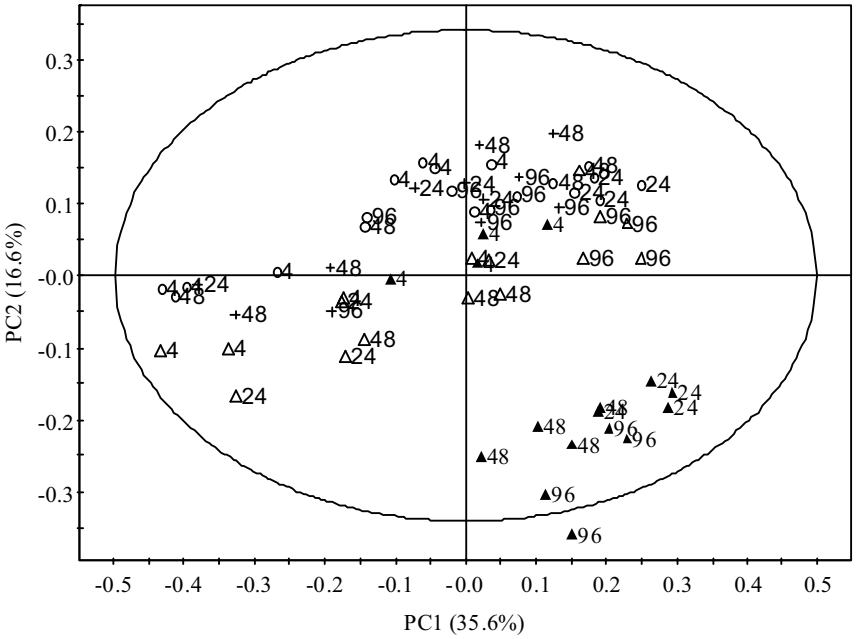
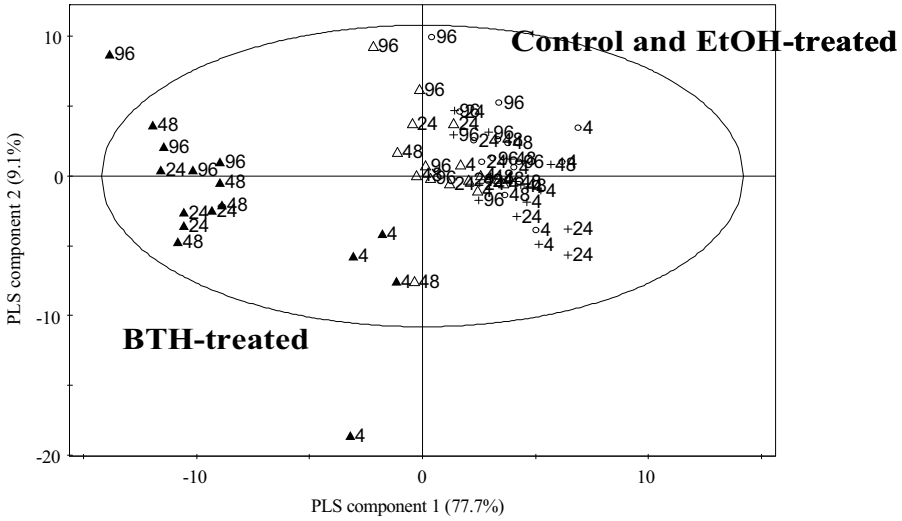


Figure 8.4. Fig. 4. Score plot of principal component analysis (PC1 vs PC2) at each time point after BTH or EtOH treatment based on ^{1}H- NMR spectra of the *Arabidopsis thaliana* (O; control 1, +; control 2, Δ; 80% EtOH-treated, ▲; BTH-treated). The eclipse represents Hotelling's T2 with 95% confidence in score plots. Numberings on the plot are the number of hours after treatment.



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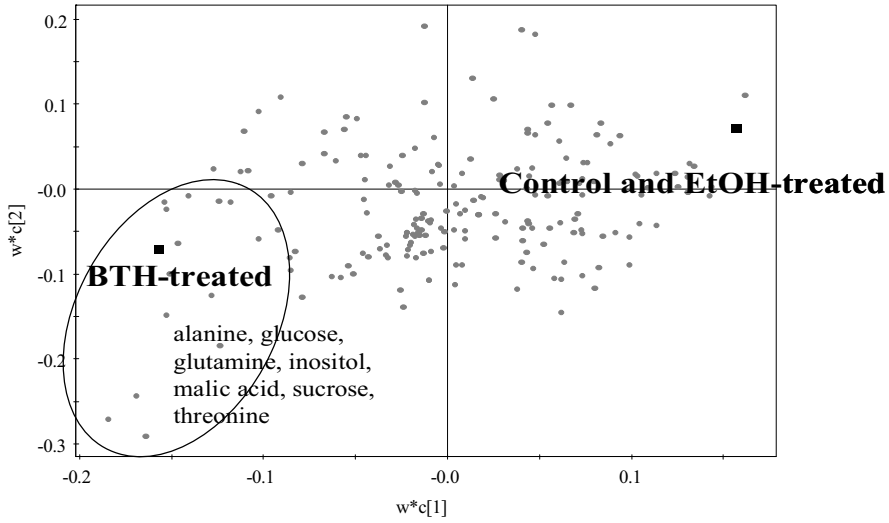
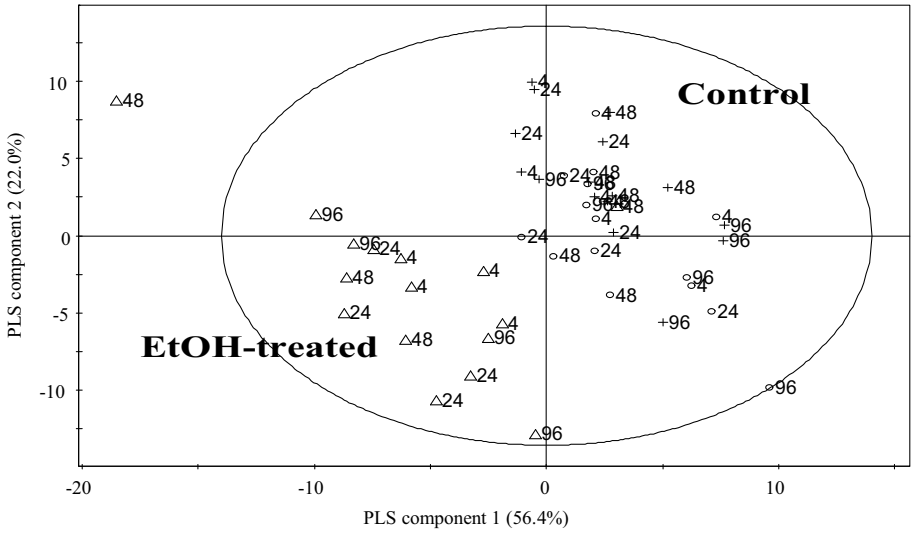
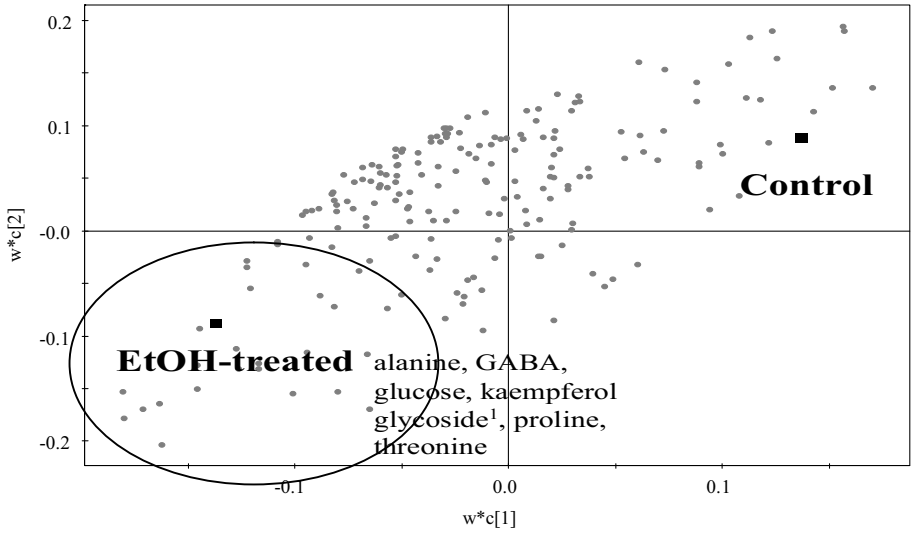


B

Figure 8.5. Score (A) and loading (B) plot of PLS-DA using two classes (class 1; non-treated and EtOH-treated, class 2; BTH-treated *Arabidopsis thaliana*). O; control 1, +; control 2, Δ; 80% EtOH-treated, ▲; BTH-treated. The eclipse represents Hotelling's T2 with 95% confidence in score plots. Numberings on the plot are the number of hours after treatment.



A



B

Figure 8.6. Score (A) and loading (B) plot of PLS-DA using two classes (class 1; non-treated class 2; EtOH-treated *Arabidopsis thaliana*). O; control 1, +; control 2, Δ; 80% EtOH-treated. The eclipse represents Hotelling's T2 with 95% confidence in score plots. Numberings on the plot are the number of hours after treatment.

8.4. Conclusions

The treatment of *Arabidopsis thaliana* with BTH induces PR-1 gene expression followed by an increase in the levels of metabolites such as glucose, glutamine, inositol, malic acid, sucrose, and threonine. This metabolic differentiation was clearly detectable using a combination of NMR spectroscopy and a supervised multivariate data analysis, PLS-DA. However, unlike the changes observed in the levels of primary metabolites, the levels of phenolic metabolites, which are generally induced by other signaling molecules like jasmonic acid or salicylic acid, did not vary. These results are consistent with the previous finding that BTH itself may prime SAR, which is then triggered by subsequent elicitation or infection. Apart from the changes resulting from BTH-treatment, it was found that the EtOH used to dissolve BTH in this study could, *per se,* act as an inducer of some metabolites. In particular, the levels of kaempferol- 3-*O*-D-glucopyranoside-7-*O*-L-rhamnopyranoside were affected by EtOH-treatment.