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Research article

Effect of benzothiadiazole on the metabolome of *Arabidopsis thaliana*

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ABSTRACT

Benzothiadiazole (BTH) is a functional analog 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 EtOH used as a solvent in this treatment may *per se* act as an inducer of the accumulation of a flavonoid.

1. Introduction

Plants interact constantly with the environment, 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 [3,6,7,16,27]. SAR can also be induced by exposing the plant to virulent, non-pathogenic 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) [19,29,31].

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 [29]. 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 [27]. 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 [15,34]. 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 [32]. 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 [31]. Different roles have been attributed to PR proteins, such as antimicrobial or antifungal activities *in vitro* activities [25,26] or the capacity of releasing elicitors [20]. 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

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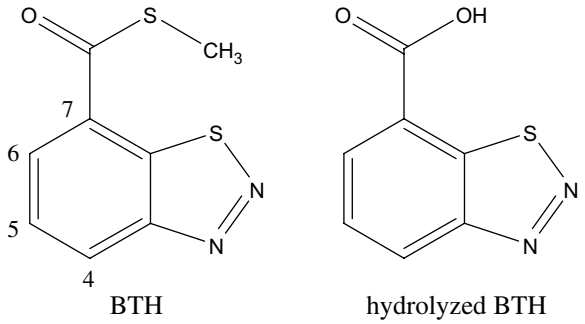

Fig. 1. Chemical structures of BTH and its hydrolyzed metabolite.

natural conditions against a broad spectrum of diseases affecting a variety of crops [8,9,21]. 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 [8,9,19]. BTH induces SAR in tobacco [8], wheat [9] and in *Arabidopsis* [21]. 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) [21]. 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 [12]. 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 [14]. A remarkable change at this level was detected only after elicitation [14], 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 [13]. 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 [11,17,28]. 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 [4,33]. 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 [5,10,24]. 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 molecular basis of systemic acquired resistance in plants.

2. Results and discussion

2.1. 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* [29]. The accumulation of the PR-1 gene in BTH-treated samples was observed 4 h after treatment and increased after 24 and 48 h, decreasing 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).

2.2. Principal component analysis of ^{1}H NMR spectra of control, EtOH- 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: MeOH-water for polar, hydrophilic metabolites and $CHCl_3$-MeOH 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 MeOH-water extract showed a great amount of amino acids, carbohydrates, organic acids, and phenolics that were clearly detected in a single spectrum [10]. 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 Fig. 2. ^{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 (Fig. 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 (Fig. 2A). 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 (Fig. 1). All known *Arabidopsis* metabolites were elucidated based on the chemical shifts and coupling constants observed which were confirmed by diverse 2D-NMR such 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 [10].

For a first overview of the metabolomic changes in BTH-treated-*Arabidopsis*, principal component analysis (PCA) was applied to the