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BTH-mediated antioxidant system responses in apple leaf tissues

Maria Skłodowska[a], Ewa Gajewska[a,*], Elżbieta Kuźniak[a], Artur Mikiciński[b], Piotr Sobiczewski[b]

[a] Department of Plant Physiology and Biochemistry, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland
[b] Szczepan Pieniążek Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland

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ABSTRACT

BTH (S-methylbenzo-1,2,3-thiadiazole-7-carbothiate), an active compound of the commercial preparation Bion, has been studied as an elicitor of resistance to fire blight (*Erwinia amylovora*) in apple. However, the biochemical mechanisms of its action are not fully elucidated. Our study indicated that BTH at the best time of its protection activity (2–14 days after application) induced changes in prooxidant–antioxidant balance in the leaves of apple trees, but in different ways in the enzymatic and nonenzymatic antioxidants. Glutathione as low molecular antioxidant as well as superoxide anion radical and lipid peroxides as oxidants exhibited changes at the early phase of BTH action. Glutathione-dependent enzymes were strongly affected by the elicitor used. On the 2nd day glutathione transferase (GST) and glutathione peroxidase (GSH-Px) activities increased by about 70% and 30% above the control, respectively. GST activity normalized about the 14th day but GSH-Px at the same time showed 27% of the control value. Among enzymes utilising hydrogen peroxide only catalase showed increase (37%) at the early phase of experiment. Compared with the control, BTH-treated plants did not show changes in ascorbate peroxidase and phenylalanine ammonia-lyase activities. Tocopherol (TOC) level diminished starting from the 7th day after BTH treatment and on the 14th day it was only 28% of the control. It is proposed that extinguishing of BTH-mediated signal resulted from TOC and glutathione action. The diminished ascorbate level at all examined times may play a crucial role in BTH-mediated cell growth regulation. The direct influence of BTH on lipid metabolism should be also taken into consideration.

1. Introduction

S-methylbenzo-1,2,3-thiadiazole-7-carbothiate (BTH) is a functional analogue of salicylic acid (SA) used to induce resistance to infectious diseases in plants (Pieterse and van Loon, 1999; Walters et al., 2005). At the molecular level BTH induces the same characteristic set of defence genes as SA, and the same spectrum of resistance reactions against fungal, bacterial and viral pathogens under field conditions but does not show a direct antimicrobial activity (Friedrich et al., 1996; Heil, 1999). It has been suggested that the activation of resistance by BTH takes place without the accumulation of SA as this compound may translocate systemically in plants by itself (Lawton et al., 1996). However, recently Umemura et al. (2009) have indicated the connection of BTH with SA metabolism. They found that BTH-treated rice exhibited increase in the activity of salicylic acid glucosyltransferase.

The exogenous application of BTH to leaves resulted in the coordinate induction of pathogen-related (PR) protein genes (Stintzi et al., 1993), enhanced the activities of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and peroxidase (POX, EC 1.11.1.7), decreased chlorophyll content (Šindelářová et al., 2002) and caused the accumulation of reactive oxygen species (ROS) (Friedrich et al., 1996).

BTH-induced resistance against fire blight disease caused by *Erwinia amylovora* has been demonstrated in pear and apple plants (Brisset et al., 2000; Maxon-Stein et al., 2002; Sparla et al., 2004). However, in bean and wheat foliar resistance-inducing application of BTH has been shown to affect negatively the host plants with respect to growth inhibition and lateral shoot formation as well as reduction of grain yield, respectively (Heil, 1999).

Production of ROS in plant tissues at the time of elicitation is under the control of NADPH oxidase and POXs (Cao and Jiang, 2006). Plant POXs have been implicated in local defence responses: the HR (hypersensitive response), lignification and in the cross-linking reactions of cell wall associated proteins such as hydroxyproline-rich or glycine-rich glycoproteins and phenolics (Kombrink and Somssich, 1995; Bolwell and Wojtaszek, 1997; Wojtaszek, 1997) as well as in systemic acquired resistance (SAR). Baysal and Zeller

Abbreviations: AA, ascorbate reduced; APX, ascorbate peroxidase; BTH, S-methylbenzo-1,2,3-thiadiazole-7-carbothiate; CAT, catalase; DHA, dehydroascorbate; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione oxidized; GST, glutathione transferase; PAL, phenylalanine ammonia-lyase; POX, peroxidase; POX-f, peroxidase utilizing ferulic acid; PR, proteins pathogenesis-related proteins; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; TBARS, thiobarbituric acid reacting substances; TOC, tocopherol.

* Corresponding author. Tel.: +48 42 635 44 16; fax: +48 42 635 44 23.
E-mail address: ewagaj@biol.uni.lodz.pl (E. Gajewska).

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(2004) indicated that POXs might have a dual role in resistance because beside the well-established defence functions they produce superoxide anion radical ($O_2^{\bullet -}$) (Bolwell and Wojtaszek, 1997), a highly toxic molecule responsible for the peroxidation of membrane lipids (Ádám et al., 1989; Bóka et al., 2007).

ROS are scavenged by both enzymatic and nonenzymatic antioxidants. One of the possible functions of ROS in defence reactions is the modulation of a redox index in cells via the main redox buffers: ascorbate and glutathione. In their reduced forms they act as hydrophilic antioxidants counteracting the deleterious effects of oxidative stress. Moreover, reduced glutathione (GSH) and reduced ascorbate (AA) have crucial roles as substrates for glutathione transferase (GST, EC 2.5.1.18), glutathione peroxidase (GSH-Px, EC 1.11.1.9) and for ascorbate peroxidase (APX, EC 1.11.1.1), respectively (Noctor and Foyer, 1998; Kuźniak and Skłodowska, 2005). These enzymes together with catalase (CAT, EC 1.11.1.6) are directly involved in ROS scavenging but they also have a broader role in governing stress responses by protecting the regulatory proteins from oxidative impairment and by redox sensing and signaling (Somssich and Hahlbrock, 1998; Öztetik, 2008; Shetty et al., 2008).

Tocopherol (TOC) is a lipophilic antioxidant with important functions in plants. It is essential to maintain membrane integrity by preventing the propagation of lipid peroxidation (Munné-Bosch, 2005) and may participate in signal transduction (Trebst, 2003; Skłodowska et al., 2009).

In apple (*Malus domestica* L.) and other *Maloideae*, BTH is often used to protect tissues against the fire blight pathogen (Eastgate, 2000; Sparla et al., 2004). Although BTH has been shown to be effective in inducing resistance, its mode of action and cellular targets are not fully elucidated. To get a further insight into the mechanism of BTH action, the BTH-induced profiles of enzymes and metabolites that might be involved in induced resistance were identified in apple tree leaf tissues. The analyses were performed 2, 7 and 14 days after elicitation, when the highest level of BTH-induced protection in apple was observed (Brisset et al., 2000). We monitored changes in the activities of antioxidant enzymes: APX, CAT, GSH-Px, GST as well as of enzymes involved in the phenylpropanoid metabolism: PAL and POX utilizing ferulic acid (POX-f) as markers of cell wall strengthening processes. The levels of $O_2^{\bullet -}$ and lipid peroxides as markers of the oxidative processes as well as the concentrations and redox ratios of ascorbate and glutathione as hydrophilic indicators and TOC as a lipophilic marker of the antioxidant status of leaf cells were also studied.

2. Material and methods

2.1. Biological material

All assays were performed on 1-year-old apple plants cv. Idared in the phase of active growth of shoots. The plants were grown in individual pots in a greenhouse at the temperature of 22 °C under natural photoperiod and humidity. They were sprayed with water solution of commercial preparation Bion 50 WG (Novartis Protection AG, Bazylea, Switzerland) containing BTH as an active ingredient. The concentration of BTH in the elicitor solution was 0.06%. The plants sprayed with distilled water were considered as the control. On the 2nd, 7th and 14th day after treatment all actively growing shoots were taken for analyses. Three fully expanded leaves (from the top) were detached from each shoot. Leaf samples (0.5 g FW) were immediately homogenized in a mortar in 5 ml ice-cold 0.05 M sodium phosphate buffer pH 7.0 containing 1 mM EDTA, 1 mM sodium ascorbate and 1% polyvinylpyrrolidone. For TOC determination the homogenate was taken and stored at −20 °C. Immediately after centrifugation (20,000 × *g*, 20 min, 4 °C) the supernatant was used for measurement of enzyme (APX, CAT, GSH-Px, GST, POX-f) activities as well as glutathione content. For lipid peroxidation determination the supernatant was stored at −20 °C. Separate homogenizations were performed to determine PAL activity and ascorbate content.

2.2. Determination of ascorbate and glutathione contents

For the determination of ascorbate content the leaves (0.5 g FW) were homogenized in 5 ml ice-cold 5% trichloroacetic acid. The modification of the colorimetric bipyridyl method of Okamura (1980), as described by Knörzer et al. (1996) was used. Total ascorbate was estimated after reduction of dehydroascorbate (DHA) to AA with dithiothreitol. The concentration of ascorbate (μmol g^{-1} FW) was determined using a calibration curve for AA as a standard. Glutathione content was determined colorimetrically using 5,5′-dithiobis-2-nitrobenzoic acid as described by Brehe and Burch (1976) and its concentration was given in nmol mg^{-1} protein. For specific assay of oxidized glutathione (GSSG) the GSH was masked by derivatisation with 2-vinylpyridine. Redox ratios for ascorbate and glutathione were calculated as {[AA]/[AA + DHA]} × 100% and {[GSH]/[GSH + GSSG]} × 100%, respectively.

2.3. Determination of TOC content

TOC content was assayed according to the method of Taylor et al. (1976). After saponification of the sample with KOH in the presence of AA TOC was extracted to *n*-hexane. The fluorescence of organic layer was measured at 280 nm (excitation) and 310 nm (emission). The concentration of TOC was expressed in μg mg^{-1} protein.

2.4. Determination of lipid peroxidation

Lipid peroxidation, estimated in terms of the concentration of thiobarbituric acid reacting substances (TBARS) was analyzed fluorometrically according to Yagi (1976). The supernatant prepared as described for the determination of enzymes (Section 2.1) was mixed with 29 mM 2-thiobarbituric acid (TBA) in 8.75 M acetic acid and heated at 95 °C for 1 h. After cooling TBARS were extracted to *n*-butanol and the fluorescence of organic layer was measured at 531 nm (excitation) and 553 nm (emission). The concentration of TBARS was estimated by referring to a standard 1,1,3,3-tetraetoxypropane. The level of lipid peroxides was expressed in nmol mg^{-1} protein.

2.5. Enzyme assays

Total GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) by the method of Habig et al. (1974). The reaction mixture contained 100 mM potassium phosphate buffer pH 6.25, 0.75 mM CDNB, 30 mM GSH and the enzyme extract. The product of CDNB conjugation with GSH absorbs at 340 nm ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). The enzyme activity was expressed in units, each representing 1 nmol of S-conjugates formed per minute per mg protein. GSH-Px activity was determined according to the method of Hopkins and Tudhope (1973) with *t*-butyl hydroperoxide as a substrate. The reaction solution contained 0.05 M potassium phosphate buffer pH 7.0, 2 mM EDTA, 0.28 μM NADPH, 4.7 μM GSH, 0.16 U glutathione reductase, 0.073 μM *t*-butyl hydroperoxide and the enzyme extract. The oxidation of NADPH was monitored by measuring absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). The enzyme activity was expressed in units, each representing 1 nmol of NADPH oxidized per minute per mg protein. APX activity was assayed following the oxidation of ascorbate to dehydroascorbate at 265 nm ($\varepsilon = 13.7\ mM^{-1}\ cm^{-1}$) by the modified method of Nakano and Asada (1981). The assay mixture consisted of 50 mM sodium phosphate buffer pH 7.0 containing 1 mM EDTA, 0.25 mM sodium ascorbate, 25 μM hydrogen