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BENZOTHIADIAZOLE, AN INDUCER OF PLANT DEFENSES, INHIBITS CATALASE AND ASCORBATE PEROXIDASE

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Key Word Index—*Nicotiana tabacum*; Solanaceae; benzothiadiazole; salicylic acid; catalase; ascorbate peroxidase; defense genes.

Abstract—Benzothiadiazole (BTH) is a recently described synthetic inducer of plant defenses. Molecular and genetic studies have suggested that it acts as a functional analogue of the endogenous defense signalling molecule salicylic acid (SA). Here we demonstrate that BTH inhibits catalase and ascorbate peroxidase, two potential targets through which SA has been proposed to act. BTH was found to be a considerably better inhibitor of catalase than SA. This is consistent with its greater potency for inducing the expression of defense-related genes, such as the acidic *PR*-1, *PR*-2 and *PR*-3 genes. In addition, induction of *PR*-1 gene expression by either BTH or SA was suppressed by antioxidants. These results suggest that changes in H_2O_2 levels or the cellular redox status may be involved in the BTH/SA-mediated activation of certain defense responses.

INTRODUCTION

Plants react to pathogen attack through a variety of active and passive defense mechanisms. At the site of infection, a hypersensitive response (HR) is often initiated in resistant plants; this appears as a necrotic lesion. Associated with the HR is containment of the pathogen. In addition, the distal uninfected parts of the plant usually develop systemic acquired resistance (SAR) which results in enhanced long-lasting resistance against the same or even unrelated pathogens [1, 2]. Both the HR and SAR are associated with increased expression of a large number of defense-related genes encoding phytoalexin biosynthetic enzymes, antiviral factors, proteinase inhibitors, peroxidases, hydrolytic enzymes, and pathogenesis-related (PR) proteins [2].

In recent years considerable progress has been made in identifying some of the signals involved in the initiation and regulation of both the HR and SAR. For example, approximately a dozen resistance genes that participate in gene-for-gene interactions, thereby initiating the activation of host defenses, have been recently cloned [3]. A number of downstream components of the defense signalling pathway(s) have also been discovered. One of the more intensely studied components is salicylic acid (SA) [2, 4, 5]. Currently, the evidence from many studies suggests that SA plays a key role in the induction and/or maintenance of plant resistance against pathogens. In tobacco, application of exogenous SA (and aspirin) induces *PR* gene expression and partial resistance to pathogens such as tobacco mosaic virus (TMV) [6]. In addition, increased levels of endogenous SA correlate with the development of resistance and the induction of *PR* genes in cucumber and tobacco [7, 8]. SA also induces the same set of genes that are activated systemically by TMV infection [9]. Furthermore, transgenic tobacco and *Arabidopsis* plants that are unable to accumulate SA due to expression of the bacterial *nahG* gene (NahG plants) fail to develop SAR and exhibit increased susceptibility to a primary infection with virulent and avirulent pathogens [10, 11].

In addition to SA, certain synthetic chemicals have been shown to induce SAR. For example, application of 2,6-dichloroisonicotinic acid (INA; Fig. 1) to tobacco induces the same spectrum of resistance and *PR* genes as is seen after SA treatment of TMV infection [9]. Since INA does not stimulate SA biosynthesis and is able to induce SAR in NahG transgenic tobacco plants, it likely mimics SA or functions at a step downstream of SA [12, 13]. Other studies have suggested that INA is a functional analogue of SA. Both SA and INA inhibit the two major H_2O_2-scavenging enzymes,

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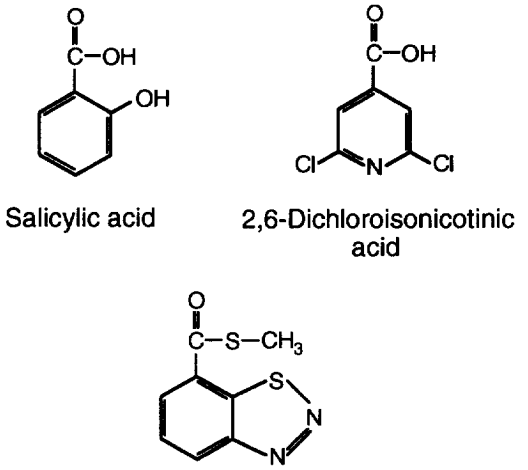

Fig. 1. Three inducers of plant defense responses.

catalase and ascorbate peroxidase (APX) [14, 15]. In addition, *Arabidopsis* mutants which fail to induce *PR* genes or develop enhanced resistance after SA treatment are also non-responsive to INA or vice versa [16–18].

Both SA and INA are moderately good inducers of resistance. However, problems with stability and phytotoxicity have prevented their use as plant protection compounds. Recently, Ryals and coworkers [19–21] have described a new synthetic crop protection agent, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Fig. 1), which is highly effective at inducing enhanced disease resistance. In tobacco and *Arabidopsis*, BTH elicits the same set of defense responses as those induced by SA. Thus, BTH and INA both appear to be functional analogues of SA. Presently, BTH is being utilized commercially as a novel type of plant protection agent. However, its mode of action and cellular targets are unknown. Since BTH induces the same set of responses as SA and INA, we have studied the effects of BTH on catalase and APX, two enzymes whose activities are inhibited by SA and INA.

RESULTS

Inhibition of Catalase and APX by BTH and SA

SA has previously been shown to inhibit catalase and APX from tobacco [14, 15, 22, 23]. Since it was suggested that BTH is a functional analogue of SA, the effect of BTH on these enzymes was tested. To determine whether tobacco APX is sensitive to BTH, crude leaf extracts were prepared, subjected to differential ammonium sulphate precipitation and analyzed for APX activity in the presence of increasing concentrations of BTH. The effect of BTH on tobacco catalase was similarly determined; however, the activity of the purified enzyme was measured. BTH was found to be an effective inhibitor of both APX and catalase (Fig. 2). To estimate the inhibitor concentration necessary for 50% inhibition (IC_{50}), catalase and APX activities were measured in the presence of different concentrations of BTH. Using double-reciprocal Lineweaver-Burk plots, substrate-dependent IC_{50} values of 48 μM for catalase and 145 μM for APX were obtained. At 500 μM, almost complete inhibition of both enzymes was achieved. For a comparison, the effect of SA on catalase and APX is also shown. While SA and BTH had similar dose-response curves for APX, BTH was a much more effective inhibitor of catalase than SA. In fact, BTH appears to be one of the most potent inhibitors of catalase reported [22, 24].

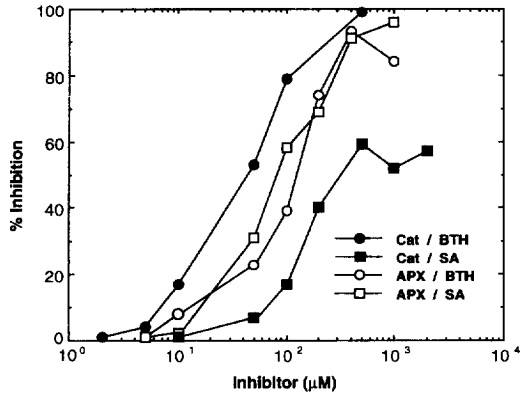

Fig. 2. Concentration-dependent inhibition of catalase and APX by SA and BTH. Values are expressed as % inhibition and represent the average of three replicated assays. The specific activities of the controls were 7.0 μmol AsA min^{-1} mg^{-1} (APX) and 34 U mg^{-1} (catalase). In the case of catalase, inhibition by SA and BTH was determined following a 1 hr preincubation in the presence of the inhibitors and a H_2O_2-generating system, consisting of glucose and glucose oxidase (H_2O_2-production was adjusted to 0.1 nmol ml^{-1} min^{-1} as analysed with the peroxidase-based method described by Mullen and Gifford [47]). The assays were performed as described in the Experimental section.

To gain insight into the mechanism through which BTH inhibits catalase activity, its kinetics of inhibition were compared to those of SA. The SA mediated inhibition of catalase requires H_2O_2 and occurs in a time-dependent manner [22]. In contrast, inhibition of catalase by BTH (100 μM) did not increase with the preincubation time; that is, it is time independent (data not shown). The presence of H_2O_2 also did not influence the effect of BTH on catalase. Furthermore, removal of BTH (by chromatography with a PD-10 column) did not restore catalase activity (data not shown). In contrast, the SA-mediated inhibition of catalase is reversible [22]. Thus, the mechanism by which BTH inhibits catalase appears to differ from that of SA, and this may explain BTH's much higher inhibitory potential. Several other catalase inhibitors such as phenols, 3-amino triazole, ascorbate, and DTT have been reported and their modes of action described (for review see Schonbaum and Chance [24]). At present, it is unclear whether BTH's mode of

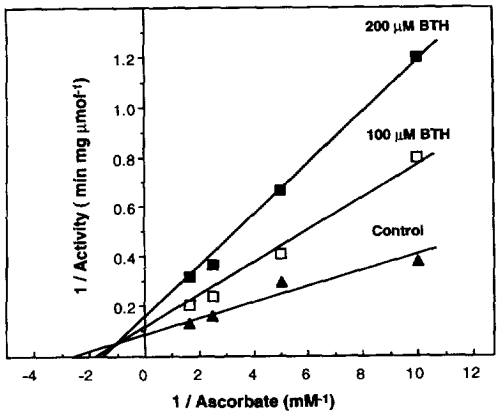

Fig. 3. Inhibition of APX by BTH at different concentrations of AsA. APX activity was measured in the presence of 0.1 to 0.6 mM AsA and 100 or 200 μM BTH. Data are shown as a double-reciprocal plot (1/APX activity vs 1/concentration of AsA, respectively). The specific activity of the control was 12.5 μmol min^{-1} mg^{-1}. Values represent the average of three replicated assays.

inhibition is similar to that utilized by any of these inhibitors.

To help elucidate the mechanism by which BTH inhibits APX, its inhibition kinetics were compared with those of SA and *p*-aminophenol. The latter is a suicide substrate of APX [25]. While *p*-aminophenol (200 μM) inactivated APX in tobacco leaf extracts in a time-dependent manner, inhibition by SA or BTH (100 μM) was time independent and was at least partially reversible ([15]; data not shown). Thus, BTH, like SA, seems to be a reversible inhibitor of this enzyme. To gain further insight into BTH's mechanism of APX inhibition, dose-response analyses with BTH were done using different concentrations of ascorbate (AsA), APX's substrate (Fig. 3). Double-reciprocal Lineweaver-Burk plots revealed mixed-type kinetics, indicating that BTH at least partially interferes with the binding of AsA at the catalytic site of the enzyme.

BTH is a more effective inducer of defense gene expression than SA

Because BTH appears to be a more efficient inhibitor of catalase than SA, we analysed BTH's ability to induce the expression of several *PR* genes (*PR*-1, *PR*-2 and *PR*-3), as well as the *SAR* 8.2 and *PAL* genes. While a 24 hr treatment of leaf discs with BTH or SA induced *SAR* 8.2 and the *PR* genes, neither compound stimulated *PAL* gene expression (Fig. 4). As anticipated, BTH was more effective than SA at inducing these genes (Fig. 4 and Table 1). Increases in *SAR* 8.2 and *PR* mRNA levels were observed after treatment with 0.1 μM BTH, and the concentration required for their half maximum induction was between 0.75 to 5 μM. In comparison, the SA concentration required for half maximum induction of these different genes ranged from 30–70 μM. No further increase in gene expression was observed at BTH and SA concentrations higher than 50 and 500 μM, respectively.

Induction of PR-1 *protein accumulation by BTH and SA is suppressed by antioxidants*

The finding that BTH, as well as SA and INA, inhibit catalase and APX suggests that these defense-activating compounds may act by blocking the activity of the two major H_2O_2-scavenging enzymes, and thereby increasing H_2O_2 levels. If elevated H_2O_2 levels or changes in the cellular redox status play a role in BTH- or SA-mediated activation of defense responses, including *PR* gene induction, then antioxidants should counteract the effect of these compounds. Antioxidants have previously been used to establish the involvement of oxidative changes, such as increases in reactive oxygen species (ROS) or alterations in redox status, in a number of important biological processes. These include activation of the transcription factor NF-κB [26–28], induction of apoptosis [29, 30], and development of amyloid β protein toxicity in neurons [31]. Thus, we tested the ability of four antioxidants, including N-acetyl cysteine (NAC), catechol, N-*t*-butyl-phenylnitrone (BPN) and nordihydroguaiaretic acid (NDGA), to inhibit the BTH- and SA-induced accumulation of PR-1 proteins. All of these antioxidants scavenge ROS. In addition, NAC, which has been used repeatedly in these types of studies, also elevates intracellular glutathione (GSH) levels by serving as an intermediate in the GSH biosynthetic pathway.

When leaf discs were treated with BTH or SA in the presence of NAC or catechol, the accumulation of PR-1 protein was substantially suppressed at 10, 14 and 24 hr after treatment in comparison to the levels detected in the leaf discs treated with BTH or SA alone (Fig. 5). Suppression by BPN and NDGA was more variable among experiments (data not shown).

DISCUSSION

Recently, BTH was shown to activate disease resistance in wheat, tobacco and *Arabidopsis* [19–21]. In tobacco, BTH treatment, as well as TMV inoculation or SA treatment result in the development of SAR. However, BTH does not increase SA levels and is able to induce disease resistance in transgenic NahG plants, which are unable to accumulate SA. Thus, it has been suggested that BTH might activate the SAR pathway by mimicking the endogenous SA signal. To further explore this possibility, we asked whether BTH can act as a functional analogue of SA and thereby interact with two proposed targets of SA, namely catalase and APX.

Previously, we proposed that the elevated levels of H_2O_2 which might result from inhibition of these enzymes would serve as a second messenger for the induction of defense responses [14, 22, 23]. However, recent studies have suggested that *PR* gene induction

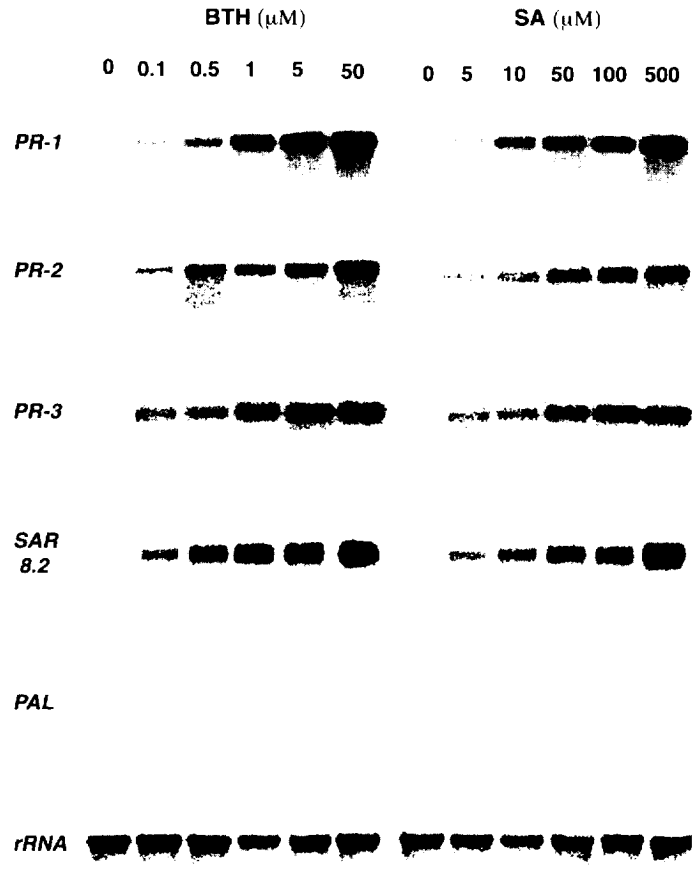

Fig. 4. Accumulation of *PR*-1, *PR*-2, *PR*-3, *SAR* 8.2 and *PAL* mRNA in response to BTH or SA treatment. Total RNA was isolated from leaf discs after a 24 hr incubation with increasing concentrations of BTH (0–50 μM) or SA (0–500 μM) and subjected to northern analysis. Blots were probed with cDNAs for the tobacco *PR*-1, *PR*-2, *PR*-3, *SAR* 8.2 and *PAL* genes. A rDNA probe was used as a standard to control for unequal gel loading. Present are the Phosphorimager scans of the RNA blots.

Table 1. Concentration of BTH and SA required for half maximum induction of several defense related genes

Genes	BTH (μM)	SA (μM)
PR-1	2.5	65
PR-2	4.5	58
PR-3	3	53
SAR 8.2	0.75	30

Leaf disc treatments and mRNA analyses were performed as described in the legend to Fig. 4. The different mRNA levels were quantified using a Phosphorimager and normalized to the rRNA signal. Estimation of the BTH and SA concentrations required for half-maximum induction of these genes was performed as described in the Experimental section.

during SAR is not activated by SA-mediated increases in H_2O_2 levels [32, 34]. Indeed, the concentration of SA in uninfected systemic tissue is probably too low to effectively inhibit catalase or APX, unless it is concentrated in a subcellular compartment [1]. Thus, SA-mediated inhibition of catalase and APX and the resulting increase in H_2O_2 do not appear to play a major role in the induction of plant defense genes in uninfected tissue. However, SA inhibition of catalase and APX and increased H_2O_2 level may play an essential role in defense responses in the inoculated leaves, where SA levels are very high. In addition, it should be noted that there is a strong correlation between the ability of many analogues of SA and INA to induce defense responses and inhibit catalase and APX [14, 15, 23]. In this context, it is particularly intriguing that a second synthetic inducer of defense responses, BTH, also inhibits both enzymes. Furthermore, BTH was found to be one of the strongest inhibitors of catalase so far reported, having a IC_{50} value of 48 μM (Fig. 2; [22, 24]). Moreover, the greater ability of BTH to inhibit catalase compared with that of SA, was consistent with its greater potency for inducing *PR* and *SAR* 8.2 genes.

The IC_{50} values of 48 μM for catalase and 145 μM for APX appear to be relatively high when compared with the concentration of BTH necessary to induce defense gene expression in the leaf discs assay (Fig. 4,

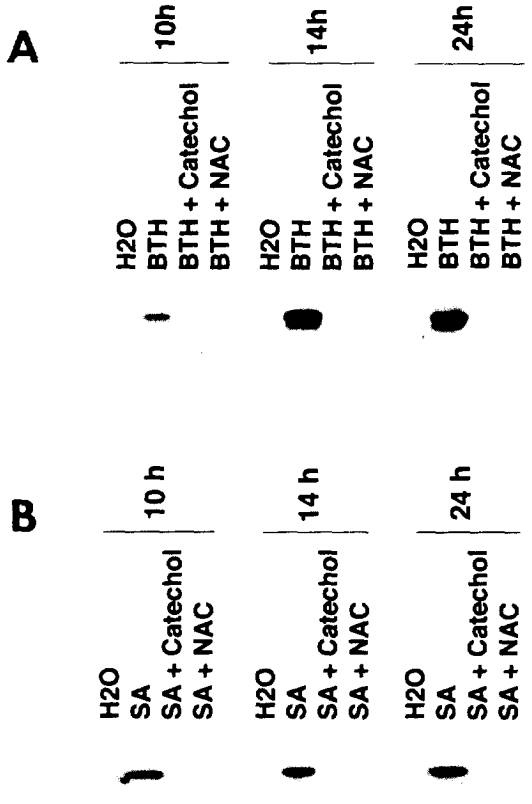

Fig. 5. Effects of catechol and NAC on BTH- or SA-induced PR-1 protein accumulation. Leaf discs were treated with 50 μM BTH (A) or 500 μM SA (B) in the absence or presence of 1 mM catechol or 20 mM NAC and harvested at the indicated times. PR-1 protein accumulation was determined by immunoblot analysis as described in Experimental procedures.

Table 1). However, these IC_{50} values are in line with the levels of BTH needed for strong induction of defense-related genes in tobacco and wheat [19, 20]. Thus, the concerted inhibitory action of BTH on the two major antioxidative enzymes, catalase and APX, may be of biological significance.

The finding that antioxidants suppress BTH- and SA-mediated induction of PR-1 protein accumulation (Fig. 5) is consistent with the hypothesis that these chemicals may act, in part, by altering cellular redox status. In contrast to our results, Green and Fluhr [35] and Friedrich *et al.* [36] reported that SA induction of *PR*-1 genes was not suppressed by NAC or catechol, respectively. In both studies, *PR*-1 gene expression was monitored relatively late (22 and 48 hr, respectively) compared to the times we analysed (10, 14 and 24 hr). Inconsistency in results among different laboratories investigating the effects of antioxidants have been seen in mammalian systems, as well as with plants and suggest the need for cautious interpretations of the results. Redox regulation of cellular defense reactions and its response to antioxidants and prooxidants is still poorly understood [37].

The structural similarities between BTH, INA and SA (Fig. 1) have previously been noted [14, 20]. All three compounds share an aromatic ring with a carboxyl group, which may be substituted at certain positions. These similarities in structure, together with the ability of BTH, INA and SA to activate identical patterns of plant defense responses, have prompted speculation as to whether these compounds share a common receptor(s) and/or effector protein(s). Interestingly, a high affinity SA-binding protein (SABP2) was recently identified which has even greater affinity for BTH than SA [38]. At present, it is unclear whether BTH/SA inhibition of catalase and APX and/or binding of SABP2 are involved in the signal transduction pathway(s) leading to activation of defense responses and disease resistance. However, regardless of whether catalase and APX play a role in the activation of resistance responses, analysis of their interaction with BTH, SA and its analogues may help define common characteristics of the BTH/SA interaction site(s). A better understanding of how BTH and SA interact with their target may facilitate the identification of other cellular factors that play critical roles in signalling plant disease resistance.

It should be noted, however, that while the spectrum of BTH targets appears to overlap with proteins that interact with SA, it might not be identical. Rüffer *et al.* [39] showed that SA binds to and inhibits aconitase, a FeS enzyme involved in the mitochondrial TCA cycle. However, while in our hands SA is a powerful inhibitor of tobacco aconitase with an IC_{50} value well below 100 μM, there was no significant inhibition by BTH (data not shown). Further studies will be necessary to determine whether BTH binding is restricted to heme proteins.

EXPERIMENTAL

Plant material. Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown at 22° in growth chambers programmed for a 14 hr light cycle at approximately 15 000 lux and were used for experimentation at 6–8 weeks.

Chemicals and enzymes. SA, ascorbic acid (AsA) and fine chemicals were from Sigma or Aldrich. BTH was obtained from Stinnes Agrar GmbH, Mühlheim, Germany.

Enzyme assays. Catalase activity was measured as previously described with a commercial oxygen electrode probe [22]. If not otherwise indicated, the reaction was started by the addition of H_2O_2 to 10 mM. Enzyme activity (i.e. O_2 production) was followed for 2 to 4 min. Catalase activity was calcd on the basis that two molecules of H_2O_2 are degraded per molecule of O_2 generated, and is expressed in Units (U) of mmol H_2O_2 decomposed per min.

APX activity was determined spectrophotometrically as described by the procedure of ref. [40]. If not otherwise indicated, APX was assayed in chelex-treated 50 mM potassium phosphate, pH 7, and 750 μM AsA. The reaction was started with 100

μM H_2O_2. The amount of protein per assay (as determined by the method of ref. [41]) was 30–50 μg. Oxidation of AsA was followed by the decrease in absorbance at 290 nm (2.8 mM^{-1} cm^{-1}). The reactions were measured for 3 min and were corrected for auto-oxidation of AsA by H_2O_2.

Protein extraction and enzyme preparation. Catalase: Tissue homogenization and catalase purification were performed as described [22]. The specific catalase activity of the purified enzyme was 34 U mg^{-1}.

Ascorbate peroxidase: Tissue homogenization was performed as described [15]. Briefly, soluble proteins were extracted with 100 mM chelex-treated potassium phosphate, pH 7, containing 1 mM AsA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 14000 *g* for 5 min and fractionated by ammonium sulphate pptn (45–85% satn). The resulting ppt. after centrifugation (14000 *g*, 15 min) was resuspended in extraction buffer and desalted on a PD-10 column (Pharmacia) equilibrated with chelex-treated 50 mM potassium phosphate, pH 7, containing 100 μM AsA. The extract was used immediately.

BTH, SA and antioxidant treatment. Tobacco leaf discs (1 cm diameter, 2 discs per 5 ml soln) were floated in Petri dishes (3.5 cm diameter) containing aq. soln of BTH (0–50 μM), SA (0–500 μM) or H_2O as control. To determine the effects of antioxidants, BTH (50 μM) and SA (500 μM) soln were supplemented with 1 mM of catechol or 20 mM of N-acetyl cysteine (NAC). After infiltration under vacuum for 2 min, the leaf discs were agitated (15 rpm) at room temp. with continuous light for 10, 14 or 24 hr.

RNA extraction and hybridization. Total RNA was extracted from leaf discs treated for 24 hr with various concn BTH or SA using the TRIzol reagent according to the suppliers instructions (GIBCO/BRL). RNA (10 μg per lane) was sepd on 1.2% agarose gels containing 1.1% formaldehyde, blotted to nylon membranes and cross-linked by UV. The following tobacco cDNA clones were used as probes for hybridization: acidic PR-1a and PR-2c [42, 43], the acidic chitinase PR-3 [44], SAR 8.2m (unpublished data) and PAL [45]. The probes were ^{32}P labelled by random priming. Hybridizations and washes were performed as described by the procedure of ref. [46]. Band intensities were quantified using a Phosphorimager. Membranes were subsequently hybridized with a tobacco rRNA probe to control for errors in gel loading. The BTH and SA concn required for half maximum induction of the different genes were estimated by defining the strongest hybridization signal on each membrane as 100% and calculating the other signals as a percentage of this signal.

Determination of PR-1 *protein level.* At various time points after vacuum infiltration, the leaf discs were homogenized in 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol and 10 μg ml^{-1} phenylmethylsulphonyl fluoride. After clarification by centrifugation (12000 *g*, 10 min), an aliquot from each of the resulting supernatants (10 μg protein) was subjected to western analysis to monitor PR-1 protein accumulation as described by the method of ref. [14].

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