β-Aminobutyric acid-mediated enhancement of resistance in tobacco to tobacco mosaic virus depends on the accumulation of salicylic acid

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A number of chemical and biological agents are known as inducers of systemic acquired resistance (SAR) to tobacco mosaic virus (TMV) in tobacco plants. In the present study, a local spray application of the non-protein amino acid DL-β-aminobutyric acid (BABA) was effective in enhancing resistance to TMV in tobacco plants containing the N gene. In contrast, the isomer γ-aminobutyric acid (GABA) was completely inactive, indicating a strong isomer specificity of aminobutyric acid in triggering enhanced virus resistance.

Rapid cell death was detected in tobacco leaf tissues after foliar application of BABA, subsequently resulting in the development of macroscopically visible, necrotic lesions. BABA-induced cell death was associated with the rapid generation of superoxide and hydrogen peroxide. As further consequences, the occurrence of lipid peroxidation in treated tissues, a local and systemic increase of salicylic acid (SA) levels and the expression of PR-1a, a molecular marker of SAR in tobacco, could be observed. None of these responses was detectable after treatment with GABA.

Enhancement of virus resistance by BABA was found to be strictly dependent on SA-mediated signal transduction since it could not be detected in salicylate hydroxylase (nahG) expressing transgenic tobacco plants. These findings suggest that in tobacco, primary processes triggered by foliar application of BABA, resemble those initiated by microbes during a hypersensitive response (HR) that result in SAR activation.

Keywords: β-Aminobutyric acid; BTH; hypersensitive response; reactive oxygen species; systemic acquired resistance; tobacco; tobacco mosaic virus.

INTRODUCTION

Natural disease resistance of plants is based on preformed and inducible mechanisms. The latter are activated in the case of pathogen contact as an active response of plant cells after recognition of the invader. Defense mechanisms can be expressed locally, at the site of pathogen attacked cells as well as systematically, in uninfected tissues. They include changes like papilla formation, cell wall lignification, production of reactive oxygen species (ROS), initiation of a hypersensitive reaction (HR), expression of pathogenesis-related proteins (PRs) and accumulation of antimicrobial metabolites [26]. In recent years, the battery of the plants' own defense reactions has attracted a lot of attention both in applied agriculture and basic research.

In this relation, scientific activities were mainly focused on the phenomenon of induced disease resistance. In general, induced resistance can be defined as an activation of mechanisms that allow plants to defend themselves against a broad spectrum of pathogens without any changes of the plant genome [57]. Depending on the activating stimulus, induced resistance can be devided in pathogen-induced systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR). In the case of SAR, SA-dependent signal transduction was found to be imperative for triggering defense responses [27, 38]. In contrast, ISR seems to operate in an SA-independent manner [39]. Essential elements of the SAR and the ISR pathway have already been characterized [15, 52].

Several natural and synthetic agents have been described as activators of defense-related processes when applied to plants and some of these may have potential application in agriculture [26, 58]. However, in only a few cases have the criteria defined by Kessmann et al. [26] been clearly demonstrated. Thus, besides an effective disease control, chemical agents should be shown to induce plant specific defense responses. At the moment, the best characterized compounds within the group of chemical activators of
With the help of transgenic tobacco and Arabidopsis plants that constitutively express the bacterial nahG gene encoding a salicylate hydroxylase, it was demonstrated that both chemicals enter the SAR signalling pathway downstream of SA [17, 53]. Meanwhile, BTH was introduced as a commercial product (Bion®, Novartis Ltd., Basel, Switzerland) in Europe for the protection of wheat against powdery mildew by means of SAR [15]. Other inducing agents like probenazole [55] or Milsana®, an extract from the giant knotweed Reynoutria sachalinensis [12], were exclusively active in only a few selected pathosystems and can therefore not be considered as effective inducers in general.

Besides BTH and INA, the non-protein amino acid DL-β-aminobutyric acid (BABA) was described as SAR inducer in several plant species belonging to the Solanaceae. BABA treatment was effective in activating plants for a successful defense against foliar diseases [7–5, 20, 47, 50] and also against soilborne pathogens [4, 36]. In addition, the inducing efficiency of BABA was recently demonstrated in cultured parsley cells, a model system for the testing of SAR-inducing agents [44]. In contrast to BABA, the isomers DL-α-aminobutyric acid (AABA) and γ-aminobutyric acid (GABA) showed only weak or no activity in parsley cell cultures [44] and in all plant–pathogen interactions tested so far [7–9, 26, 47, 56]. This indicates a high isomer specificity of aminobutyric acid in defense activation.

In tomato, Cohen and coworkers were able to correlate BABA-induced resistance to Phytophthora infestans with the accumulation of several PR proteins in treated and in non-treated parts of the plants [45]. When tested in vitro, BABA exhibited no direct activity against various pathogens [7–5, 26, 47, 50] which is a further important prerequisite for abiotic inducers of disease resistance [26]. However, except for these biochemically based studies on BABA-activated mechanisms, the mode of action of this resistance-inducing chemical was not further examined in detail. In the present study the classical SAR test system tobacco-tobacco mosaic virus (TMV) was used to gain an insight into primary BABA-induced processes in plants.

**MATERIALS AND METHODS**

**Materials**

BTH was supplied by K.-L. Nau (Novartis Ltd., Frankfurt, Germany). BABA was purchased from Fluka (Buchs, Switzerland), all other chemicals came from Sigma (Deisenhofen, Germany). Tobacco PR-1a c-DNA was provided by L. Friedrich (Novartis Ltd., Research Triangle Park, U.S.A.).

**Plants**

Seeds of transgenic PR-1a:uidA tobacco plants [16] were provided by D. F. Klessig (Rutgers University, NJ, U.S.A.), seeds of transgenic NahG-10 tobacco [16] came from K. Lawton (Novartis Ltd., Research Triangle Park, U.S.A.). Non-transgenic tobacco (Nicotiana tabacum cv. Xanthi-nc), bearing the N gene derived from Nicotiana glutinosa for hypersensitive resistance to TMV, was used for inducer verification. Tobacco plants were grown under controlled conditions in a 14 h/10 h day/night cycle at 24°C and 18°C, respectively.

**Inducer treatment**

Chemical treatment was performed by spraying aqueous solutions of the aminobutyric acids (10 mm) on leaves 3 and 4 of tobacco plants with six fully developed leaves. BTH (0.5 mm) was applied in the form of the commercial plant activator Bion® (WP 50, Novartis Ltd., Frankfurt, Germany). For biological induction of resistance, corresponding leaves of tobacco plants were inoculated with TMV (10 μg ml⁻¹ in 25 mm sodium phosphate buffer pH 7.0) using celite (0.1 mg ml⁻¹) as abrasive.

**Challenge inoculation with TMV**

Control plants and inducer-treated tobacco plants were challenge inoculated on leaf 5 with TMV (10 μg ml⁻¹ in 25 mm phosphate buffer pH 7.0). Since N gene tobacco is genetically resistant to TMV by local lesion formation, evaluation of inducer effects on these plants was performed 7 days after inoculation by counting the number of local lesions as well as by measuring the diameter of 20 lesions on each leaf. In this system, effective inducers mediate an enhancement of resistance by reducing the symptom severity [17].

**Quantification of SA**

Total contents of SA (the sum of free and conjugated SA) in tobacco leaves were determined according to Molders et al. [37] with some modifications. For SA extraction, tobacco leaf material (1 g) was grinded in liquid nitrogen and subsequently homogenized in a mortar after addition of 5 ml MeOH (90% v/v). The homogenate was centrifuged for 15 min at 4°C with 15000 rpm and the resulting supernatant carefully removed. After resuspension of the pellet in 5 ml MeOH (90% v/v) and a further centrifugation, both methanolic supernatants were combined and vacuum-reduced to 1/10 of the original
volume. Extracts were directly hydrolysed for 2 h at 80°C in the presence of 10 mM HCl to release glucosidically bound SA. After acidic hydrolysis, pH of the extract was adjusted to 6.5 with 1 M NaOH. Total SA-contents were analysed by HPLC (Pharmacia, Uppsala, Sweden) using a fluorescence detector (LC 304, Linear Instruments, Reno, U.S.A.). Recovery rates were determined by adding SA standards to blank samples. SA levels of test samples were respectively corrected for recovery.

Analysis of PR-1a gene expression

Expression of PR-1a was checked in wild-type tobacco \( \langle \text{Nicotiana tabacum cv. Xanthi-nc} \rangle \) by Northern gel blot analysis. Total RNA was extracted from tobacco leaf discs (100 mg FW) which were collected from inducer-treated and non-treated leaves according to Kästner et al. [25]. RNA (10 µg) was separated on 1.1% (w/v) agarose gels which contained 6% (v/v) formaldehyde as described previously [E4]. Equal loading of samples was verified by adding ethidium bromide (0.01 mg ml\(^{-1}\)) in the sample buffer. RNA was blotted overnight onto a positively charged nylon membrane (Hybond-N\(^{+}\), Amersham Life Science, Buckinghamshire, U.K.) by capillary transfer using 10 x SSC (1 x SSC = 0.15 mM sodium chloride, 0.015 mM sodium citrate pH 7). After transfer, RNA was covalently bound to the membrane by using a u.v. cross-linker (Amersham Life Science).

Prehybridisation was carried out for 4 h at 47°C in 2 x SSC, 5 x Denhard’s solution [1 x Denhard’s = 1 mM EDTA (pH 8.0)], 0.02% (v/v) each of polyvinyl-pyrolidone, Ficoll and bovine serum albumin, 1% (v/v) formamide, sonicated salmon sperm DNA (100 µg ml\(^{-1}\)) and 1% (w/v) SDS. \(^{32}\)P-labelled cDNA probes were synthesized by PCR amplification of insert DNA present in a pBluecript vector (Stratagene, La Jolla, U.S.A.) by using the primer OL 7 (5'GGT GGC GGC TCT AG-3') and OL 53 (5'-GTA AAA CGA CGG CCA GTG AGC-3'). A highly labelled probe was obtained by running \(^{32}\)P-dATP (10 mCi ml\(^{-1}\); Amersham Pharmacia Biotec, Freiburg, Germany) in each PCR sample. Hybridization to labelled PR-1a cDNA was carried out for 16 h at 47°C. The blots were then washed 3 x for 15 min at 65°C with 1 x SSC containing 1% (w/v) SDS. After air-drying, blots were exposed to X-ray film at -80°C.

As a second approach to demonstrate PR-1a gene activation after inducer treatment, the expression of a chimeric PR-1a:\(\text{uidA}\) reporter gene in transgenic tobacco plants was tested. Activity of \(\beta\)-glucuronidase (GUS) was determined according to Conrath et al. [40]. The fluorescence of 4-methylumbelliferone (MU) was measured with a spectrofluorometer (Hitachi F 2000).

\(\beta\)-Aminobutyric acid-mediated enhancement of resistance to tobacco mosaic virus

**Evaluation of cell death, callose formation and lipid peroxidation**

Histochemical monitoring of cell death was carried out by infiltrating Evans blue in tobacco leaf discs according to Schraudner et al. [42]. As a second method, leaf discs were stained with trypan blue to visualize cell death as described previously [25]. For further microscopical evaluation of BABA- and TMV-mediated lesion formation, leaf discs were heated for 3 min in a mixture of lactic acid, phenol and water (1:1:1, v/v/v). After cooling, the discs were destained for 2 days in the same mixture [42].

Formation of callose was determined by staining leaf discs with aniline blue according to Dietrich et al. [14].

Lipid peroxidation was estimated by measuring the accumulation of thiobarbituric acid reactive substances (TBARS) spectrophotometrically as described by Rusterucci et al. [46]. The content of lipid peroxides was determined by using the molar extinction coefficient for malondialdehyde (1.56 x 10\(^{5}\) M\(^{-1}\) cm\(^{-1}\)).

**RESULTS**

**BABA enhances resistance to TMV in tobacco**

The aminobutyric acid isomers AABA, BABA and GABA were tested for their potential to enhance resistance to TMV in tobacco plants that contain the \(N\) gene. As a positive control, the chemical activator BTH was included which was already demonstrated as a highly effective inducer in tobacco [17]. Compared to the untreated control, plants which were sprayed on lower leaves either with 10 mM BABA or 0.5 mM BTH, exhibited significantly smaller lesions on upper leaves after challenge inoculation with TMV (Table 1). Furthermore, the number of lesions was considerably reduced in treated plants (data not shown). Foliar application of BABA at concentrations below 5 mM resulted in a markedly reduced efficacy, at 0.5 mM BABA was completely inactive (data not shown). In contrast to BABA, a treatment of tobacco plants with 10 mM GABA did not enhance resistance to TMV (Table 1).
**Table 1.** Effects of treatments with BTH, AABA, BABA or GABA on the expression of resistance in tobacco plants (Xanthi-nc) to TMV. Resistance was assessed by inoculating an upper leaf (leaf 5) with TMV (10 µg ml⁻¹), 7 days after application of the inducers to leaf 3 and 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TMV lesion size (mm) ± SD</th>
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<tbody>
<tr>
<td>None (control)</td>
<td>1.55 ± 0.24</td>
</tr>
<tr>
<td>BTH (0.5 mm)</td>
<td>0.25 ± 0.11</td>
</tr>
<tr>
<td>AABA (10 mm)</td>
<td>1.10 ± 0.16</td>
</tr>
<tr>
<td>BABA (10 mm)</td>
<td>0.53 ± 0.30</td>
</tr>
<tr>
<td>GABA (10 mm)</td>
<td>1.47 ± 0.18</td>
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*Values are the means ± SD of five experiments with five plants per treatment.

**Fig. 1.** Northern Blot analysis of total RNA extracted from tobacco (Xanthi-nc) leaves sprayed with 10 mM BABA, 10 mM GABA or 0.5 mM BTH. Seven days after inducer application, RNA from treated leaves (lanes 1, 3, 5 and 7) as well as from non-treated leaves (lanes 2, 4, 6 and 8) was separated by electrophoresis and probed with 32P-labelled tobacco PR-1a cDNA.

1). GABA was ineffective up to 50 mM (data not shown). The application of AABA (10 mM) resulted in slightly reduced TMV lesion sizes after challenge inoculation (Table 1). Due to its weak activity, AABA was not applied in the further experiments.

**Fig. 2.** Expression of a chimeric PR-1a:uidA reporter gene in treated (leaf 4) and non-treated (leaf 5) parts of transgenic tobacco plants. GUS activity was determined 7 days after application of 0.5 mM BTH, 10 mM BABA or 10 mM GABA to leaf 3 and 4. *Means from four experiments (five plants per treatment) significantly different from control (P < 0.05).

**BABA induces local and systemic PR-1a gene expression**

Induction and maintenance of resistance in tobacco is closely associated with the coordinated activation of SAR genes [54]. Genes coding for proteins of the PR-1 group are highly expressed after treatments with chemical and biological inducers [54]. In tobacco plants, foliar application of 10 mM BABA resulted in the induction of PR-1a mRNA in the treated as well as in non-treated (systemic) leaves (Fig. 1). As expected, BTH (0.5 mm) also induced local and systemic PR-1a expression. No accumulation of PR-1a mRNA was detectable after application of 10 mM GABA (Fig. 1).

As an additional experimental approach, transgenic tobacco plants containing a chimeric PR-1a:uidA reporter gene construct were used to demonstrate the activation of the PR-1a promotor by aminobutyric acid derivatives. As shown in Fig. 2, maximal GUS activity was detected in leaves sprayed with BTH which was used as a positive control. Lower but significantly induced activities were determined in leaves treated with BABA. In contrast, almost no GUS activity was measured in control plants and in plants sprayed with GABA. However, increased GUS activities were also evident in non-treated leaves of tobacco plants induced by a local application of BTH or BABA, indicating a systemic activation of the PR-1a promotor by both chemicals (Fig. 2).

**BABA mediates local cell death**

Foliar application of BABA to tobacco plants resulted in the appearance of HR-like lesions on the treated leaves. Lesions were macroscopically visible 24 h after inducer treatment, especially on the adaxial leaf surface. Lesions subsequently increased in size within the next 24 h (not shown). Compared to the formation of local lesions after TMV inoculation of tobacco plants bearing the N gene, a greater number of smaller lesions appeared in BABA-treated tissues (Fig. 3). In contrast to BABA, no visible signs of cell death were detectable after GABA application (not shown).

Staining of tobacco leaf material with trypan blue and Evans blue indicated a rapid initiation of cell death by BABA. Starting 14–16 h after inducer application, localized blue spots, indicating irreversible uptake of Evans blue and therefore cell death, were detected in BABA-treated tissues (Fig. 4(a)). Blue coloured regions expanded up to 48 h after inducer treatment (Fig. 4(b)). Microscopical evaluation of leaf discs stained with trypan blue, revealed clusters of dead cells surrounded by apparently unaffected cells (Fig. 4(c)). Besides cell death,
Fig. 3. Lesion phenotype on tobacco leaves provoked by various inducer treatments. (a) Phenotype of necrotic lesions, 6 days after application of BABA (10 nm) or TMV (10 μg ml⁻¹) inoculation. (b) Microscopical appearance of lesions in destained tobacco leaf tissue, 6 days after application of BABA (10 nm) or TMV (10 μg ml⁻¹) inoculation.
a staining of leaf discs with aniline blue showed a rapid deposition of callose around dying cells [Fig. 4(d)].

**Histochemical localization of BABA-mediated generation of ROS**

Occurrence of cell death is expected to be closely related to the generation of ROS [33]. For this reason, histochemical staining methods for the detection of O$_2^-$ and H$_2$O$_2$ were performed. Infiltration of NBT revealed a short period of O$_2^-$ generation at 6–8 h after BABA application [Fig. 5(a)]. Subsequently, production of H$_2$O$_2$ was detectable by DAB staining by 12–14 h after inducer application [Fig. 5(a)]. Staining continued increasing up to 48 h after inducer application. The spatial distribution of O$_2^-$ and H$_2$O$_2$ production was more or less diffuse [Fig. 5(a)], whereas H$_2$O$_2$ accumulation was distinctively localized in regions where cell death could be detected by staining with Evans blue [Figs 4(b) and 5(c)].

**BABA induces lipid peroxidation**

Since cell death resembling an HR was detected as a consequence of foliar BABA application, lipid peroxidation in tobacco leaf tissues was verified as a further typical marker related to cell death. The extent of membrane damage in control leaves and BABA-treated leaves was estimated with the thiobarbituric acid assay. BABA-induced lipid peroxidation was evident by increased TBARS contents, measurable 48 h after inducer application. Compared to control plants, a three-fold higher TBARS level was determined as maximal response at 96 h after BABA treatment [Fig. 6].

**BABA mediates local and systemic accumulation of SA**

SA is known to play an important role in the process of SAR activation by HR-inducing microbes [27]. To estimate whether the occurrence of BABA-mediated cell death could be correlated with an accumulation of SA, the content of total SA in tobacco plants was determined. Increased SA levels were found in the leaves 7 days after spraying with BABA [Fig. 7(a)]. As previously described in the literature [17, 33], an inoculation with TMV caused a massive accumulation of SA whereas SA contents did not increase in tobacco plants treated with the plant activator BTH [Fig. 7(a)]. Similarly, SA accumulated in the upper (systemic) leaves of BABA-treated tobacco plants but at a lower level than in directly treated leaves. Again, SA contents measured in systemic leaves of BABA-treated and TMV-preinoculated plants were much higher than in control and BTH-treated plants [Fig. 7(b)].

**SA accumulation as prerequisite for BABA-mediated enhancement of virus resistance**

Activation of SAR by HR-inducing microbes depends on the accumulation of SA [16] whereas chemical activators like BTH and INA induce SAR in an SA-independent manner [17, 33]. Transgenic tobacco that constitutively expresses a salicylate hydroxylase gene (nahG) from *Pseudomonas putida* cannot accumulate significant amounts of SA [18]. This transgene is a helpful tool to demonstrate the involvement of SA in SAR expression. Since BABA was found to induce SA accumulation in wild type tobacco (Fig. 1), transgenic NahG plants were used to test the relationship between BABA-induced enhancement of TMV resistance and SA accumulation. For comparison, non-transgenic tobacco plants and the well characterized inducers BTH and TMV were included in this set of experiments. As shown in Fig. 8 and already known from the literature [17], BTH enhanced resistance in wild type Xanthi-nc as well as in the transgenic line NahG-10, whereas TMV was exclusively active as inducer in Xanthi-nc and not in NahG-10. Interestingly, BABA treatment resulted in the same phenotype of resistance as seen in the case of the biotic induction by TMV. BABA as an effective inducer in non-transgenic tobacco, exhibited no activity in transgenic NahG plants (Fig. 8). A quantitative evaluation of the plants revealed a significant reduction of disease symptoms in wild type and transgenic tobacco by BTH compared to the control, but no enhanced resistance in TMV-preinoculated or BABA-treated NahG tobacco plants (Fig. 9). These results indicate that BABA-mediated enhanced resistance to TMV in tobacco is SA-dependent. In addition, it is interesting to note that BABA-mediated lesion formation was still evident in NahG tobacco (not shown).

**DISCUSSION**

It is expected that chemical inducers of disease resistance will play the key role for the integration of the SAR concept in modern plant protection strategies [31]. For this reason, the search for new inducing agents as well as the elucidation of the mode of action of already available inducers has intensified in recent years [36]. Indeed, only a limited number of inorganic, natural organic or synthetic compounds with a generally accepted resistance-inducing activity are known so far [26, 45]. However, even in case of the best studied inducers, INA and BTH, little knowledge exists about their mode of action and the cellular receptors or target molecules with which they interact [44]. The resistance inducing potency of BABA has also been documented in a number of studies [7–9, 20, 47, 50]. The highest efficacy of the compound was demonstrated in several members of the Solanaceae. Therefore, tobacco was used in the present study to
β-Aminobutyric acid-mediated enhancement of resistance to tobacco mosaic virus

Fig. 4. Histochemical detection of cell death by Evans blue/trypan blue staining and of callose formation by anilin blue staining in BABA-treated tobacco leaves. Trypan blue-stained leaf discs were examined by light microscopy, anilin blue-stained discs by fluorescence microscopy. (a) Control (left); Evans blue-stained leaf disc, 16 h after application of 10 mM BABA (right). (b) Control (left); Evans blue-stained leaf disc, 48 h after application of 10 mM BABA (right). (c) Trypan blue staining, 48 h after application of 10 mM BABA. (d) Anilin Blue staining, 24 h after application of 10 mM BABA.

Fig. 5. Histochemical localization of ROS in tobacco leaf tissue following application of 10 mM BABA. For the detection of $O_2^-$, leaf discs were vacuum-infiltrated with NBT. $H_2O_2$ accumulation was demonstrated by DAB infiltration. (a) NBT staining, 8 h after BABA application. (b) DAB staining, 12 h after BABA application. (c) DAB staining, 48 h after BABA application.

examine biochemical and molecular mechanisms involved in BABA-induced resistance. In the pathosystem tobacco- TMV, BABA-mediated enhancement of virus resistance could be demonstrated. In contrast, the aminobutyric acid derivative GABA was completely inactive, the isomer AABA had a much lower activity indicating a high isomer specificity of aminobutyric acid in triggering enhanced virus resistance. Similar results have been obtained in tomato, pepper and sunflower [8, 20, 47, 50]. In a more related study, using the system tobacco-Peronospora tabacina, only BABA but not AABA and GABA was found to induce resistance against blue mold [7]. Thus, data available at the moment suggests a very specific interaction of BABA with unknown cellular components in treated plant tissues. As a further characteristic of plant activation by BABA, relatively high concentrations of the chemical were needed to trigger resistance in almost all systems
tested so far. Foliar applied concentrations of BABA in the millimolar range (1–20 mM) were usually utilized to achieve plant protection [6, 26, 47]. In addition, effective BABA concentrations used for inducing resistance in plants by root-drenching were 5–40 mM [4, 26, 36, 56]. Compared to BABA, a much lower concentration (0.5 mM) of the plant activator BTH was sufficient for the enhancement of resistance in tobacco to TMV, indicating differences in the uptake and/or in the mode of action of both inducers.

Activation of certain marker genes encoding PR proteins is a common feature associated with resistance induction. Thus, in tobacco, PR 1-a gene expression could be demonstrated locally and systemically after foliar BABA application. This is in agreement with the results obtained by immunoblot analysis of extracts from tobacco plants sprayed with BABA, where PR-1 protein accumulation was already shown [7]. In addition, BABA-induced resistance in tomato and pepper was also found to be correlated with the induction of PR-proteins [6, 22]. However, no PR protein accumulation could be detected in tobacco after stem injection of BABA although the plants were strongly protected against *Peronospora tabacina* [7]. This indicates that different processes may be activated by BABA in tobacco depending on the location of inducer application. A foliar spray treatment with BABA triggers PR gene expression and virus resistance, whereas a stem application seems to activate yet unknown mechanisms which mediate resistance against the blue mold pathogen but were not related to PR protein accumulation. This view is further supported by the observation that a stem application of BABA did not enhance virus resistance in tobacco (Siegrist et al., unpublished results).

The occurrence of cell death as a rapid reaction following application of BABA was an unexpected result
Fig. 8. Phenotype of TMV lesions in wild type (Xanthi-nc) tobacco (upper row) and in transgenic NahG tobacco (lower row), 1 week after challenge inoculation with TMV (10 μg ml⁻¹) on leaf 5. Inducers [0.5 mM BTH, TMV (10 μg ml⁻¹), 10 mM BABA] were applied to lower leaves (leaf 3 and 4), 7 days before challenge inoculation.

Fig. 9. Effect of various treatments [0.5 mM BTH, TMV (10 μg ml⁻¹), 10 mM BABA] on the expression of resistance in wild-type tobacco plants (Xanthi-nc) and in transgenic NahG tobacco against TMV. Resistance was assessed by inoculating an upper leaf (leaf 5) with TMV (10 μg ml⁻¹), 8 days after application of the inducers to leaf 3 and 4. *Means from three experiments (5 plants per treatment) significantly different from control (P < 0.05).

obtained in the present study. Indeed, a critical review of the literature dealing with BABA-induced resistance revealed that this HR-resembling reaction of plant tissues to foliar application of the chemical was already noticed by other authors, but was not discussed in relation to plant defense activation [7, 8, 47]. Moreover, it was also observed that AABA, which exhibited a slight but reproducible activity in the tobacco/TMV system (Table 1), induced cell death when applied to tobacco leaves. However, compared to BABA, lesion formation occurring after AABA treatment was markedly less pronounced (Siegrist et al., unpublished results).

Further studies from our lab provide additional evidence for BABA-mediated cell death in plants. In tomato, foliar application of the inducer resulted in SAR expression against Phytophthora infestans [25]. In this system, the plant phenotype observed after BABA treatment was nearly identical to that appearing in the case of a biotic SAR induction mediated by local lesion forming pathogens like tobacco necrosis virus [2, 25]. Furthermore, it was recently demonstrated that BABA also causes cell death in the roots of tomato plants after a soil drench application leading to resistance against Fusarium oxysporum f.sp. lycopersici [4]. This kind of plant response prompted us to examine how BABA-induced cell death could be linked to the enhancement of virus resistance in tobacco and the processes subsequently observed. As the first detectable reaction, a generation of ROS was evident, indicating an initial interaction of BABA with a cellular target which directly or indirectly provokes an oxidative burst. The nature of this interacting component is still unknown and awaits further studies. It could be localized
in the apoplast or in cell wall-associated regions, since a small portion of radiolabelled BABA remained in the cell wall of treated tomato leaves whereas no binding to cytoplasmatic proteins could be detected [6].

Besides ROS generation, lipid peroxidation was detected in tobacco tissues, providing further evidence for typical cell death-aligned responses initiated by BABA. However, increased TBARS contents were not obvious until 2 days after inducer treatment when necrotic lesion formation was already macroscopically visible. The reason could be the low sensitivity of the thiobarbituric acid assay that does not allow detection of small changes in already damaged membranes at earlier stages.

In the case of a biotic induction of disease resistance, localized cell death and the formation of HR-lesions resulting in defense gene activation are characteristically triggered by viruses and microbes [45]. For this reason, we compared typical responses that appeared after treatment of tobacco plants with the biotic inducer TMV and the chemical inducer BABA. In agreement with already published data [35], local and systemic accumulation of SA was evident in tobacco after TMV inoculation. Interestingly, we could also find increased SA levels in treated and in non-treated parts of tobacco plants sprayed with BABA, indicating an involvement of SA in BABA-mediated enhancement of TMV resistance. This assumption was further supported in experiments with NahG plants. As expected, BTH was found to operate independently of SA. Whereas, enhancement of resistance was blocked in plants induced by TMV and BABA. From these results, it can be concluded that BABA triggers mechanisms in tobacco leaf tissues which are typical for biotic inducers of resistance acting by local lesion formation. Therefore, under our experimental conditions, BABA seems to imitate a pathogen attack. However, the formation of necrotic lesions in BABA-treated NahG tobacco indicates that primary processes initiated by this inducer are in no way affected by the low SA level of the transgenic plants.

Apart from microbial initiation of cell death, known lesion-mimic mutants of certain plants display necrotic lesions that resemble those of an HR, and exhibit activated defense mechanisms as well as SAR. Intensively studied examples are cell death mutants from Arabidopsis thaliana which express spontaneous lesion formation in the absence of pathogens [14]. These mutants showed elevated concentrations of SA, constitutive expression of PR proteins and enhanced resistance against virulent pathogens [14, 36]. More recently, lesion-mimic mutants of rice have been described which were resistant against the blast fungus Magnaporthe grisea [46]. A further line of evidence for cell death-associated activation of SAR without any microbial stimulus can be deduced from studies with lesion-mimic transgenic plants. For example, tobacco and potato plants that constitutively express a bacterio-opsin proton pump displayed necrotic lesions, elevated SA levels, increased PR gene expression and enhanced resistance against pathogens [1, 36]. Further transgenic, lesion-forming plants with similar properties have been described [13].

Other chemicals and natural compounds, besides BABA, are known to induce resistance in plants by triggering HR-like responses. In potato, a spray treatment with arachidonic acid generated massive local lesion formation and subsequently SAR against Phytophthora infestans [5, 33] and Alternaria solani [11]. In this system, a local but not systemic accumulation of SA was demonstrated [11]. Nevertheless, by using NahG potato plants, SA was found to play an essential role in arachidonic acid-mediated SAR [39].

Other examples of cell death-inducing agents are chemicals like the herbicide Paraquat or the dye Rose Bengal, that act by producing ROS when applied to plants as foliar sprays. Both chemicals have been demonstrated as effective SAR inducers in tobacco [16, 46]. Furthermore, inorganic phosphate salts which induce SAR in different plants were recently described to operate by causing ROS generation, cell death and SA accumulation [38]. Similar results have been obtained after application of elicitors, proteins excreted by Phytophthora spp. [24]. Cell death-associated activation of defense genes and SA accumulation is also known from tobacco plants treated with ozone or exposed to u.v.-light [57].

In the case of BABA-mediated lesion formation, as well as in most of the above mentioned examples, a complex regulation seems to be involved that link cell death initiation and the succeeding processes leading to disease resistance. In contrast, cell death caused by unspecific damage of plant tissues, e.g. dry ice treatment, did not activate SA synthesis and plant defense against pathogens [32; Siegrist et al., unpublished results].

In conclusion, our data provide evidence that foliar application of the chemical inducer BABA to tobacco plants results in the activation of mechanisms resembling those initiated by necrotizing microbes and viruses that trigger SAR.

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β-Aminobutyric acid-mediated enhancement of resistance to tobacco mosaic virus

REFERENCES


106

J. Siegrist et al.


