Physiological and Molecular Plant Pathology (1997) **51**, 305–322

Accumulation of β-1,3-glucanase and chitinase isoforms, and salicylic acid in the DL-β-amino-n-butyric acid-induced resistance response of pepper stems to *Phytophthora capsici*

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(*Accepted for publication August 1997*)

Pepper (*Capsicum annuum* L.) plants sprayed with DL-β-amino-n-butyric acid (BABA) were protected against *Phytophthora capsici* infection. BABA treatment induced the synthesis and accumulation of β-1,3-glucanases and chitinases in the stem tissues of pepper plants. Their accumulation was very pronounced in the stems challenge-inoculated with *P. capsici* after BABA treatment. Several β-1,3-glucanase and chitinase isoforms accumulated in BABA treated *P. capsici*. When analysed by immunoblot of the denatured proteins, the 20 kDa β-1,3-glucanase and 32 kDa chitinase were found in pepper stems treated with BABA and/or infected by *P. capsici*. BABA treatment did not stimulate capsidiol production in pepper stems, but prior treatment led to high accumulation in *P. capsici*-infected ones. Unlike capsidiol production, BABA treatment triggered a dramatic increase in the endogenous levels of salicylic acid (SA) in pepper stems. The increase in endogenous SA was much pronounced in *P. capsici* infected stems after BABA treatment. In conclusion, the induction of resistance to *P. capsici* in pepper plants by BABA treatment positively correlated with the accumulation of certain β-1,3-glucanase and chitinase isoforms, and SA. These results suggest strongly that SA may act as an endogenous signal responsible for activating particular components of resistance to *P. capsici* and the induction of pathogenesis-related proteins such as β-1,3-glucanase and chitinase.

INTRODUCTION

In plants infected with necrosis-inducing pathogens, the development of symptoms is accompanied by the *de novo* synthesis of one or more new proteins. These proteins are called pathogenesis-related (PR) proteins and are likely related to plant resistance against fungal pathogens [*1*, *3*, *4*, *15*, *16*, *19*, *20*, *22*, *25*, *30*, *38*]. It has also been reported that several chemicals, such as salicylic acid, 2,6-dichloroisonicotinic acid, and amino-n-butyric acids not only induced resistance in plants against pathogens [*18*], but also elicited the accumulation of PR proteins [*2*, *8*, *23*, *31*, *42*, *46*, *47*, *48*].

Asselin *et al*. [*2*] and Lotan and Fluhr [*23*] showed that amino-n-butyric acids induced the accumulation of PR proteins in tobacco leaves. In recent years, the accumulation of PR proteins such as β-1,3-glucanases, chitinases, and P14a has also

Abbreviations used in text: BABA, DL-β-amino-n-butyric acid; Glc-NAc, *N*-acetyl-glucosamine; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PR, pathogenesis-related; SA, salicylic acid; TCA, trichloroacetic acid.

0885-5765/97/110305+18 $25.00/0/pp970119

been detected in tomato plants treated with DL-β-amino-n-butyric acid, together with the induction of resistance to *Phytophthora infestans* [*7*, *8*]. In contrast, these PR proteins did not accumulate or accumulated to a much lower level in DL-α or γ-amino-n-butyric acid-treated plants. It was reported that DL-β-amino-n-butyric acid protected tobacco against blue mold (*Peronospora tabacina*) via a mechanism not related to PR protein accumulation, because the compound did not induce PR-1 or β-1,3-glucanase and only slight accumulation of chitinase [*7*].

PR proteins such as β-1,3-glucanase and chitinase appear to have *in vitro* antifungal activity alone or in combination [*25*, *45*]. Extracellular chitinases would have an initial role in limiting pathogen growth upon entry of hyphae into the host, while vacuolar chitinases could have a secondary or delayed effect following cell lysis [*25*]. Like chitinases, β-1,3-glucanases may also indirectly trigger defence reactions within the plant, because oligosaccharides, released from the fungal cell wall by the action of these hydrolases, could act as elicitors of the biosynthetic pathways that lead to the accumulation of phytoalexins [*17*, *37*]. The accumulation of phytoalexins may lead to the development of resistance following infection by pathogens. In previous studies we have demonstrated that the production of the phytoalexin capsidiol in either the resistant or metalaxyl-treated pepper plants was correlated with the control of phytophthora blight [*11*, *12*, *13*]. Lotan and Fluhr [*23*] also reported that DL-α-amino-n-butyric acid induced the production of capsidiol in tobacco leaves.

Salicylic acid (SA) has been suggested to be an endogenous signal molecule for induced resistance, because its concentration rises dramatically following infection with necrosis-inducing pathogens in cucumber, tobacco, and *Arabidopsis* [*10*, *24*, *27*, *32*, *34*, *43*]. The accumulation of SA in pathogen-infected leaves is correlated with the induction of PR proteins and resistance [*24*, *27*, *43*, *48*] but the extent to which PR proteins play a role in SA-mediated resistance is still not fully determined.

By using polyacrylamide gel electrophoresis and isoelectric focusing, we have shown previously that some PR proteins such as β-1,3-glucanase and chitinase were induced and accumulated in pepper stems after inoculation with *Phytophthora capsici*, more distinctly in incompatible than in compatible tissues [*14*, *19*]. Our observations in these studies provided strong support for the possible implications of the two hydrolases in disease resistance. However, all the isoforms of the two hydrolases did not seem to be associated with disease resistance. Some isoforms such as the acidic β-1,3-glucanase isoform Ga 2 were important in resistance expression, whereas most basic isoforms of β-1,3-glucanases were involved in pathogenesis.

In the present study, pepper plants treated with DL-β-amino-n-butyric acid (BABA) and/or inoculated with *P. capsici* were analysed for the accumulation of pathogenesis-related (PR) proteins, the phytoalexin capsidiol, and salicylic acid. We further examined whether or not PR proteins, capsidiol, and salicylic acid could be involved in resistance induced in pepper plants by BABA.

MATERIALS AND METHODS

Plant and fungus

Pepper (*Capsicum annuum* L.) cv. Hanbyul was used in the studies. Pepper seeds were sown in a plastic tray (55 × 35 × 15 cm) containing a steam-sterilized soil mix (peat moss, perlite, and vermiculite (5:3:2. v/v/v), mixed together with sand, and loam

(1:1:1, v/v/v). Four pepper seedlings at the four-leaf stage were transplanted into small plastic pots ($5 \times 15 \times 10$ cm) containing the peat moss mix, sand, and loam (1:1:1·2, v/v/v). Pepper plants were raised in a growth room at 25 ± 3 °C which provided 5000 lux illumination for 16 h per day.

All experiments were done with the virulent isolate S197 of *Phytophthora capsici*. The fungus was grown on oatmeal agar plates at 28 °C in the dark for 7 days and then induced to sporulate under fluorescent light at 28 °C. Zoospores were induced to release by incubating the culture plates in sterile water for 40 min at 4 °C then 30 min at room temperature. The zoospores were collected by filtering through two layers of cheesecloth and their concentration adjusted to 10^5 zoospores per millilitre using a hemacytometer.

Inducer treatment, challenge inoculation, and disease assessment

Pepper (cv. Hanbyul) plants at first-branch stage were uniformly sprayed with 1000 μg ml^{-1} of an aqueous solution of DL-β-amino-n-butyric acid (BABA) (Sigma) using a glass atomizer. The stems of plants were wounded by making 1 cm longitudinal slits in the upper, middle and bottom regions 4 days after treatment with BABA. A small quantity of sterile cotton soaked in a zoospore suspension (1×10^5 ml^{-1}) of *P. capsici* S197 was placed on the three wounded sites of each stem. The inoculated sites were then covered with plastic tape to maintain moist conditions. Segments of stem from the inoculated regions were harvested at different times after mock-inoculation and *P. capsici* inoculation and then stored at −70 °C until analysed.

Disease severity of phytophthora blight in pepper plants was rated daily after inoculation of *P. capsici* based on a 0–5 scale, as previously described [*39*].

All data are the means of 10 inoculated plants. All experiments were repeated with similar results. Data are presented from one experiment only.

Preparation of stem extracts

Liquid nitrogen frozen pepper stems were homogenized in 0·5 M sodium acetate buffer (pH 5·2) containing 15 mM 2-mercaptoethanol using a pre-chilled mortar and pestle. The crude extracts were centrifuged at 10000 ***g*** for 60 min at 4 °C. The supernatant was then centrifuged at 20000 ***g*** for 60 min at 4 °C. The clear supernatants were collected and their protein contents were assayed according to the method of Bradford [*6*] using bovine serum albumin as a standard.

Proteins in crude extracts were precipitated by mixing with four volumes of acetone at −20 °C overnight. The precipitate was collected by centrifugation at 15000 ***g*** for 15 min, washed twice with cold acetone, and dried. The residue was resuspended in 30 mM sodium acetate buffer (pH 5·2). The suspensions were centrifuged at 15000 ***g*** for 15 min to clarify them and then stored at −70 °C.

Measurement of β-1,3-glucanase activity

β-1,3-Glucanase activity in the crude stem extracts was measured by a colorimetric assay of Kauffmann *et al.* [*16*] using laminarin (Sigma) as substrate. The assay measured the reducing sugars formed from laminarin by the β-1,3-glucanase activity contained in the extracts. The substrate buffer was 0·1 M sodium acetate buffer (pH 5·2) containing laminarin (1 mg ml^{-1} buffer). The reaction mixture contained 0·9 ml substrate buffer and 0·1 ml enzyme solution (stem extract). The reaction tubes

were incubated at 37 °C for 1 h. The resulting reducing sugars were measured using the method of Nelson [*28*]. Glucose was used as a standard. One katal (kat) was defined as the enzyme activity catalysing the formation of 1 mol glucose equivalents s^{-1}.

Measurement of chitinase activity

Chitinase activity in the crude stem extracts was measured by a colorimetric assay [*36*]. The reaction mixture contained 0·5 mg washed chitin and various volumes of enzymatic solution in a final volume of 0·5 ml 0·1 M sodium acetate (pH 5·2). The mixture was incubated in a shaking water bath at 37 °C for 1 h. After incubation, the mixture was centrifuged at 12000 ***g*** for 30 min. For the determination of chitinase, 0·3 ml of the supernatant was incubated at 37 °C with 5 µl of 25 % glucuronidase to hydrolyse the chitin oligomers. The amount of liberated *N*-acetyl-glucosamine (Glc-NAc) was determined as described by Reissig *et al.* [*36*] with the following modifications. The 0·1 ml of 0·6 M potassium tetraborate was then added to the above tubes before heating for 3 min in a boiling water bath. After cooling, 1 ml of reagent diluted 1 : 2 with glacial acetic acid was added and incubated at 37 °C for 20 min. The reagent stock solution contained 10 % (w/v) 4-(dimethylamino)benzaldehyde in a mixture of glacial acetic acid and 11·5 M HCl, 87·5 ml : 12·5 ml (v/v). The resulting Glc-NAc was determined using the method of Legrand *et al.* [*22*]. Chitinase activity was expressed in katals. One katal (kat) was defined as the enzyme activity catalysing the formation of 1 mol of Glc-NAc s^{-1}. Chitinase activity was determined from a calibration curve previously described by Boller *et al.* [*5*].

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF)

Polyacrylamide gel electrophoresis of proteins under native conditions was performed using 15 % (w/v) polyacrylamide separating gels and 5 % stacking gels. Anodic polyacrylamide gels were buffered with 1·5 M Tris-HCl (pH 8·8) according to Davis [*8*]. Cathodic polyacrylamide gels were buffered with 0·3 M potassium acetate buffer (pH 4·3) according to Reisfeld *et al.* [*35*]. The gels were run at 4 °C at a constant current of 5 mA with bromophenol blue and pyronine Y as tracking dyes, respectively.

Isoelectric focusing of proteins in stem extracts was carried out on 10 % polyacrylamide gel containing ampholines (pH 3·0–10, Sigma) according to the manufacturer's protocol (LKB). The pI markers, ranging from pI 3·6 to 9·3 (Sigma), were coelectrophoresed to estimate the pI of the various proteins. Samples were loaded in the centre of the IEF gel and the voltage was increased stepwise: 200 V for 30 min, 400 V for 1 h, 600 V for 1 h, 800 V for 1 h, 1000 V for 1 h, and 1200 V for 1 h. The IEF gel was fixed in 20 % (v/v) trichloroacetic acid (TCA) for 1 h and then briefly rinsed with distilled water. The gel was stained with Serva blue W and then destained in 0·02 % (v/v) TCA until background stain disappeared.

The separation of each sample using polyacrylamide gel electrophoresis and isoelectric focusing was repeated three to five times.

Detection of β-1,3-glucanase isoforms on the gel

After electrophoresis, the PAGE gels were equilibrated in 50 mM sodium acetate (pH 5·2) for 20 min. The gels were then incubated at 37 °C for 60 min in 25 mM sodium acetate (pH 5·2) containing 0·5 % laminarin. The gels were washed three times with

distilled water after incubation. The gels were then put into a glass tray containing 0·15% 2,3,5-triphenyl tetra-zolium chloride in 1 M NaOH. The tray was kept in a boiling water bath for 5 min, until red bands appeared. To reduce the pink background, the gel was put into 7·5% (v/v) acetic acid as soon as the bands had developed [*29*].

Detection of chitinase isoforms on the overlay gels after the PAGE and IEF

After electrophoresis or isoelectric focusing, both gels attached to supporting glass plates were equilibrated in 0·1 M sodium acetate (pH 5·2) for 10 min. The resolved gels were then covered with 7·5% (0·75 mm thick) polyacrylamide overlay gels (attached to another supporting plate) containing 0·04% (w/v) glycol chitin in 0·1 M sodium acetate (pH 5·2). The liquid between the PAGE or IEF gels and overlay gels was eliminated by gently rolling a test tube over the surface of the separating gel. The sandwich gels were incubated at 37 °C for 2 h in a plastic container under moist conditions. Overlay gels were then incubated in freshly prepared 0·01% (w/v) fluorescent brightener 28 in 0·5 M Tris-HCl (pH 8·9) at room temperature for 5 min [*41*]. The brightener 28 solution was discarded and the overlay gels were destained in distilled water at room temperature in the dark for 2 h. Chitinase isoforms were visualized as dark zones by placing the overlay gels on an UV transilluminator and were photographed. The overlay gels with chitinase isoforms were stored in a refrigerator.

Immunoblotting of PR proteins after electrophoresis

Before immunoblotting, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 15% SDS polyacrylamide gels with an overlaid stacking gel of 5%, according to the method of Laemmli [*21*]. Molecular weight markers ranging from 14·4 to 97·4 kDa (Bio-Rad) were co-electrophoresed at a constant current of 5 mA to estimate molecular weights of the various proteins.

For immunodetection, the proteins separated in the SDS-polyacrylamide gel were electroblotted onto a nitrocellulose membrane (0·45 μm pore size) for 6 h in a transfer buffer containing 50 mM Tris, 380 mM glycine, 0·1% (w/v) SDS, and 20% (v/v) methanol (pH 7·5) at a constant current of 200 mA [*40*]. After transfer of proteins, the blots were washed three times at 5 min intervals with phosphate-buffered saline (PBS) and then blocked for 2 h in PBS containing 3% bovine serum albumin (IgG Free) and 0·02% sodium azide. Without incubation in the blocking solution, the protein markers ranging from 14·4 to 97·4 kD (Bio-Rad) were stained in a solution containing 0·1% amidoblack, 25% isopropanol, and 10% acetic acid and then destained in a solution containing 25% isopropanol and 10% acetic acid. After blocking, the blots were washed twice, 5 min each, with PBS, and incubated for 5 h with primary antibodies diluted with PBS. Antisera against β-1,3-glucanase, chitinase, and PR-1 proteins of tomato were provided by Dr. Joosten, Wageningen Agricultural University, Wageningen, The Netherlands. The blots were rinsed four times at 5 min intervals with PBS, and then equilibrated twice in a solution containing 150 mM NaCl and 50 mM Tris (pH 7·5). A goat anti-rabbit IgG(H+L)-alkaline phosphatase conjugate (BRL) (diluted 1:5,000) was added to the blots for a 1 h incubation, followed by washing four

times at 5 min intervals with PBS. Antigens were then visualized after incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BRL), as substrates for alkaline phosphatase reaction. When the bands had clearly appeared, after 10–15 min, the reaction between enzyme and substrate was stopped with PBS containing 20 mM EDTA.

Capsidiol determination

Two grams of pepper stem tissues were placed in 250 ml Erlenmeyer flasks with 15 ml g^{-1} fresh weight of 40 % aqueous ethanol and then vacuum-infiltrated. The flasks were stoppered and placed on a reciprocal shaker at approximately 110 strokes min^{-1}. After 5 h the tissues were removed by filtration and the filtrates vacuum-concentrated at 40 °C to approximately one-half volume. The concentrate was partitioned twice with ethyl acetate. The ethyl acetate fractions were dehydrated with $MgSO_4$ and evaporated to dryness at 40 °C. Following the transfer of the residue to vials with peroxide-free diethyl ether and evaporation of the ether, the dry residue was dissolved in 0·5 ml of ethyl acetate and then stored at −20 °C until assayed. To analyse for capsidiol, the ethyl acetate sample was dissolved in a solution of methyl myristate (4 mM) in ethanol, the ester serving as an internal standard. Capsidiol was assayed by gas-liquid chromatography according to Hwang and Sung [*13*]. Equal fractions (2–3 μl) from each ethanol solution were injected into a Packard model 419 gas liquid chromatograph fitted with a glass column (182 cm long, 2 mm inside diameter) containing Gas Chrom Q (80–120 mesh) coated with 3 % SE 30. The column was kept at 162 °C, the injector at 192 °C, and the flame ionization detector at 230 °C. The carrier gas was nitrogen at a 40 ml min^{-1} flow rate with hydrogen and air at 30 and 300 ml min^{-1}, respectively. Retention times were 12·9 and 15·0 min for methyl myristate and capsidiol, respectively. Capsidiol in the samples was identified by cochromatography with authentic capsidiol, which was provided by Dr. A. Stössl, Agricultural Canada, Research Center, London, Ontario, Canada.

Salicylic acid determination

One gram of stem tissue was ground in 2·5 ml of 90 % methanol using a pre-chilled motar and pestle. The extract was centrifuged at 12000 ***g*** for 15 min. The pellet was resuspended in 100 % methanol and re-extracted at 12000 ***g*** for another 15 min. Supernatants from both extractions were combined and dried under N_2. The residue was resuspended in 2·5 ml of 5 % (w/v) trichloro-acetic acid and filtered. The filtrate was partitioned with 5 ml of a 1:1 (v/v) mixture of ethyl acetate/cyclopentane containing 1 % (v/v) isopropanol. The top organic phase was harvested, and then dried under N_2. The residue was resuspended in 0·5 ml of 23 % methanol in 20 mM sodium acetate (pH 5·0). Salicylic acid was quantified by fluorescence using HPLC as described previously [*33*, *48*]. Fifty microlitres were injected onto a LiChrosorb RP-18 8-μm guard column (4·6 mm × 1·5 cm) linked to a LiChrosorb RP-18 8-μm C-18 column (4·6 mm × 25 cm), maintained at 40 °C. Salicylic acid was separated isocratically with 23 % (v/v) methanol in 20 mM sodium acetate buffer (pH 5·0) at a flow rate of 1 ml min^{-1}. Salicylic acid concentrations in samples were determined with a HPLC spectrofluorescence detector (Waters) equipped with a 200 watt xenon-mecury continuous arc lamp and a 360 nm cut-off filter. An excitation wave length was

313 nm and an emission wave length was 405 nm, as described by Raskin *et al.* [*33*]. The limit of detection was 10 ng of salicylic acid per gram fresh weight. Quantitation was determined over the linear range (10–1000 ng ml^{-1}) of the calibration curve for salicylic acid sodium salt (Sigma).

RESULTS

Effect of BABA on phytophthora blight

The disease progress curves for pepper (cv. Hanbyul) plants inoculated with the isolate S197 of *P. capsici* 4 days after treatment with 1000 μg ml^{-1} BABA at first-branch stage, or ones inoculated only with *P. capsici*, are shown in Fig. 1. Disease symptoms appeared

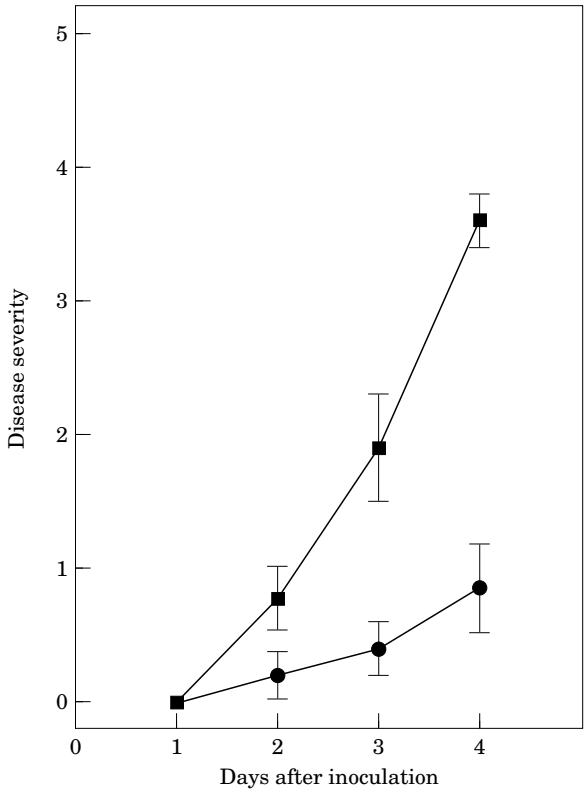

Fig. 1. Protection of pepper plants (cv. Hanbyul) at the first-branch stage against *P. capsici* infection by DL-β-amino-n-butyric acid. Plants were uniformly sprayed with 1000 μg ml^{-1} of the compound and challenge-inoculated 4 days later on pepper stems. Each bar represents a mean ± standard deviations from three replicates. (■) *Phytophthora capsici*; (●) BABA + *P. capsici*.

2 days after inoculation. Disease development was strikingly reduced in pepper plants treated with BABA before challenge inoculation, so that about 75% protection was seen. Two days after inoculation, the surface of the inoculated pepper stems began to become somewhat brownish and slightly sunken. Inoculation with *P. capsici* caused a dark brown discolouration and severe rot in the pepper stems, accompanied by a

sudden wilt of the entire plants. However, disease spread slowly in pepper plants treated with BABA before challenge inoculation. In the BABA-treated plants, dark brown stem lesions were restricted to the inoculated area.

Induction of β-1,3-glucanase and chitinase activity by BABA

β-1,3-Glucanase and chitinase activities were measured in the stem extracts from control (untreated and uninoculated) pepper plants, ones treated only with BABA, ones inoculated only with *P. capsici* S197, and ones inoculated with *P. capsici* after treatment with BABA (Fig. 2). β-1,3-Glucanase and chitinase activities remained at a

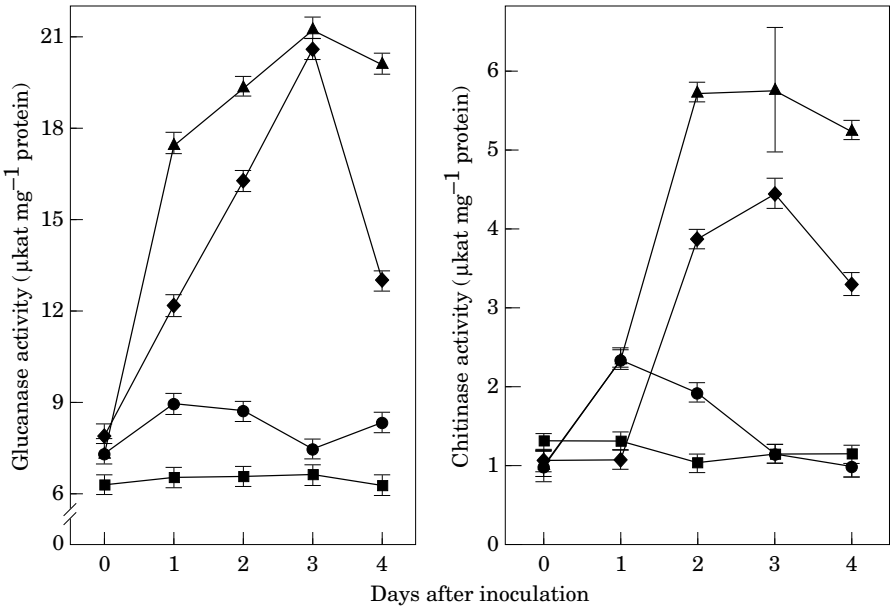

FIG. 2. Time courses of β-1,3-glucanase and chitinase activity in stem extracts from pepper (cv. Hanbyul) plants treated with 1000 μg ml^{-1} of DL-β-amino-n-butyric acid and challenge-inoculated 4 days later with *P. capsici* at the first-branch stage. Each bar represents a mean ± standard deviations from three replicates. (■) Untreated; (●) BABA; (◆) *P. capsici*; (▲) BABA + *P. capsici*.

low level in healthy control pepper stems. In pepper stems treated with BABA, their activity increased significantly as compared to that in the healthy control pepper stems. Accumulation of the two hydrolytic enzymes also was remarkably stimulated in the infected pepper stems, reaching maximum levels 3 days after inoculation. In particular, their activities occurred to a higher extent in challenge-inoculated pepper stems after BABA treatment.

β-1,3-Glucanase isoforms in BABA P. capsici *interactions*

Acidic β-1,3-glucanase isoforms were detected in the extracts of BABA-treated and/or inoculated pepper stems collected at various times after challenge inoculation with *P. capsici* by using 15 % native PAGE gels run with anodic buffer systems (Fig. 3). The

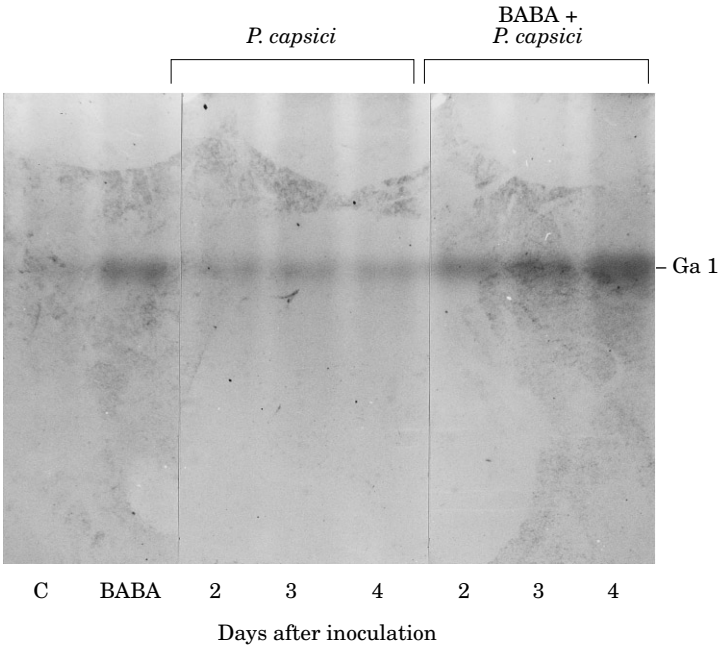

FIG. 3. Detection of acidic β-1,3-glucanase isoforms after anodic native polyacrylamide gel electrophoresis of stem extracts from pepper (cv. Hanbyul) plants treated with 1000 μg ml^{-1} DL-β-amino-n-butyric acid and/or 4 days later challenge-inoculated with *P. capsici* at the first-branch stage. Crude enzyme extracts (1 μg protein) were loaded on 15% PAGE gels and stained for β-1,3-glucanase isoforms.

intensity of isoform bands could be considered the specific activity of individual β-1,3-glucanase isoforms, because the same amounts of crude protein preparations were loaded on the same PAGE gel, as previously described by Kim and Hwang [*19*]. Only one acidic β-1,3-glucanase isoform (designated Ga 1) was detected in BABA-treated and/or inoculated pepper stems. However, isoform Ga 1 occurred at low levels in the control (untreated and uninoculated) pepper stems. The activity of the acidic isoform Ga 1 was very high in pepper stems treated only with BABA, or BABA-treated and inoculated. In the latter pepper stems, its activity increased continuously from 2 days after challenge inoculation.

Chitinase isoforms in BABA P. capsici *interactions*

Acidic and basic chitinase isoforms in the extracts of BABA-treated and/or inoculated pepper stems were detected at various times after challenge inoculation with *P. capsici* (Fig. 4). Three acidic chitinase isoforms (designated Ca 1, Ca 2, and Ca 3) and eight basic chitinase isoforms (designated Cb 1, Cb 2, Cb 3, Cb 4, Cb 5, Cb 6, Cb 7, and Cb 8) were detected in the BABA-treated and/or infected pepper stems. Only three isoforms, Ca 1, Ca 3, and Cb 2, remained at low levels in healthy control and BABA-treated stems. However, isoform Ca 2 accumulated to a relatively high level in pepper stems after BABA treatment. The activities of Ca 1 and Ca 3 were higher in the infected stems after BABA treatment than without BABA treatment. In particular,

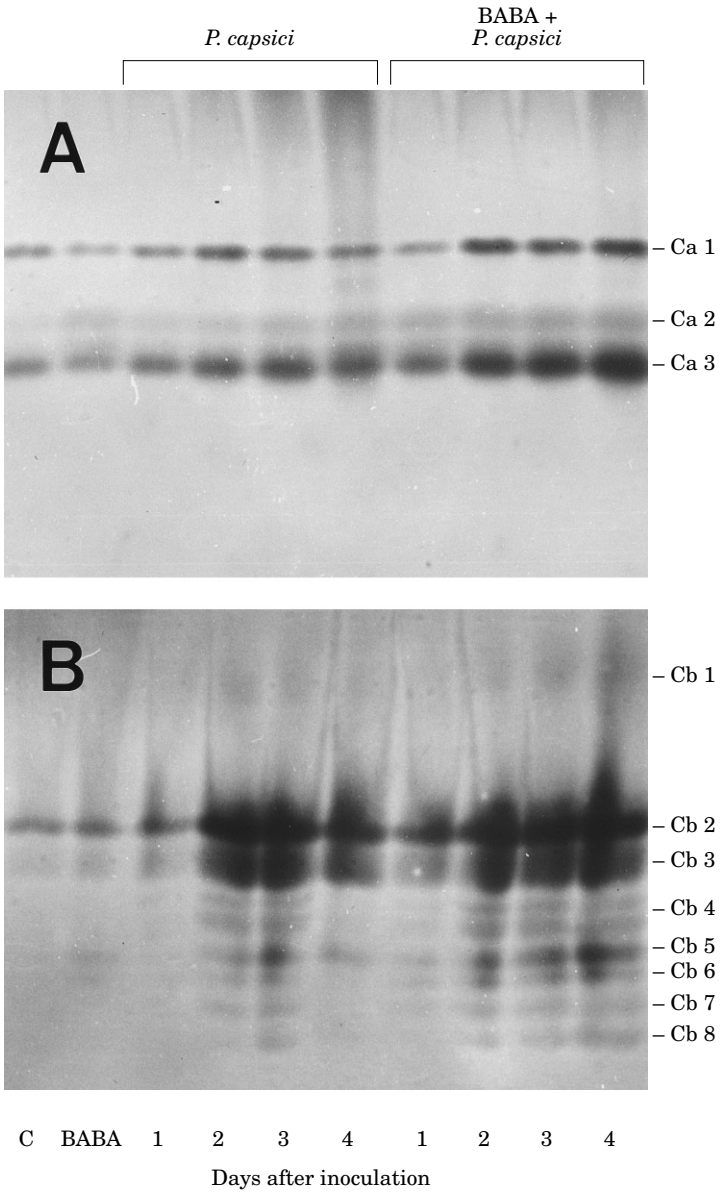

FIG. 4. Detection of acidic and basic chitinase isoforms on overlay gel after anodic (A) and cathodic (B) native polyacrylamide gel electrophoresis of stem extracts from pepper (cv. Hanbyul) plants treated with 1000 μg ml^{-1} DL-β-amino-n-butyric acid and/or 4 days later challenge-inoculated with *P. capsici* at the first-branch stage. Crude enzyme extracts (1 μg protein) were loaded on 15% PAGE gels.

induction and accumulation of basic isoform Cb 3 was more pronounced in the infected stems, irrespective of BABA-treatment. The other basic chitinase isoforms were induced to relatively high levels in the BABA-treated and/or infected stems.

To determine the isoelectric points of the chitinase isoforms induced by BABA

treatment and/or *P. capsici* infection, crude enzymes from the pepper stems extracted at 4 days after inoculation were isoelectric-focused on 10% IEF gels with a pH range of 3·0–10 (Fig. 5). Four acidic (pIs 3·6, 4·1, 4·2 and 6·4) and seven basic (pIs 7·2, 8·6,

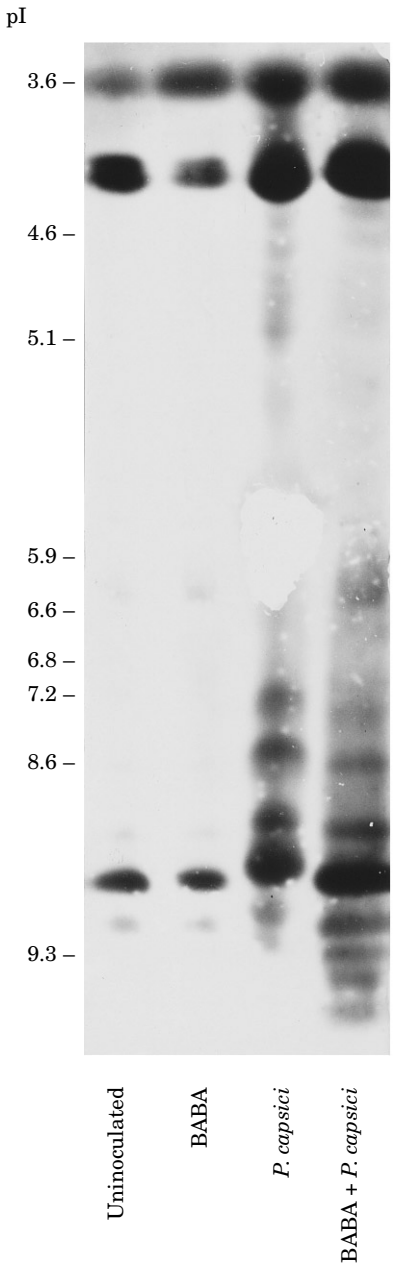

FIG. 5. Detection of chitinase isoforms on an overlay gel after isoelectric focusing (pH 3–10) of stem extracts from pepper (cv. Hanbyul) plants treated with 1000 μg ml^{-1} DL-β-amino-n-butyric acid and/or 4 days later challenge-inoculated with *P. capsici* at the first-branch stage. Crude enzyme extracts (5 μg protein) were loaded on 10% isoelectric focusing gels.

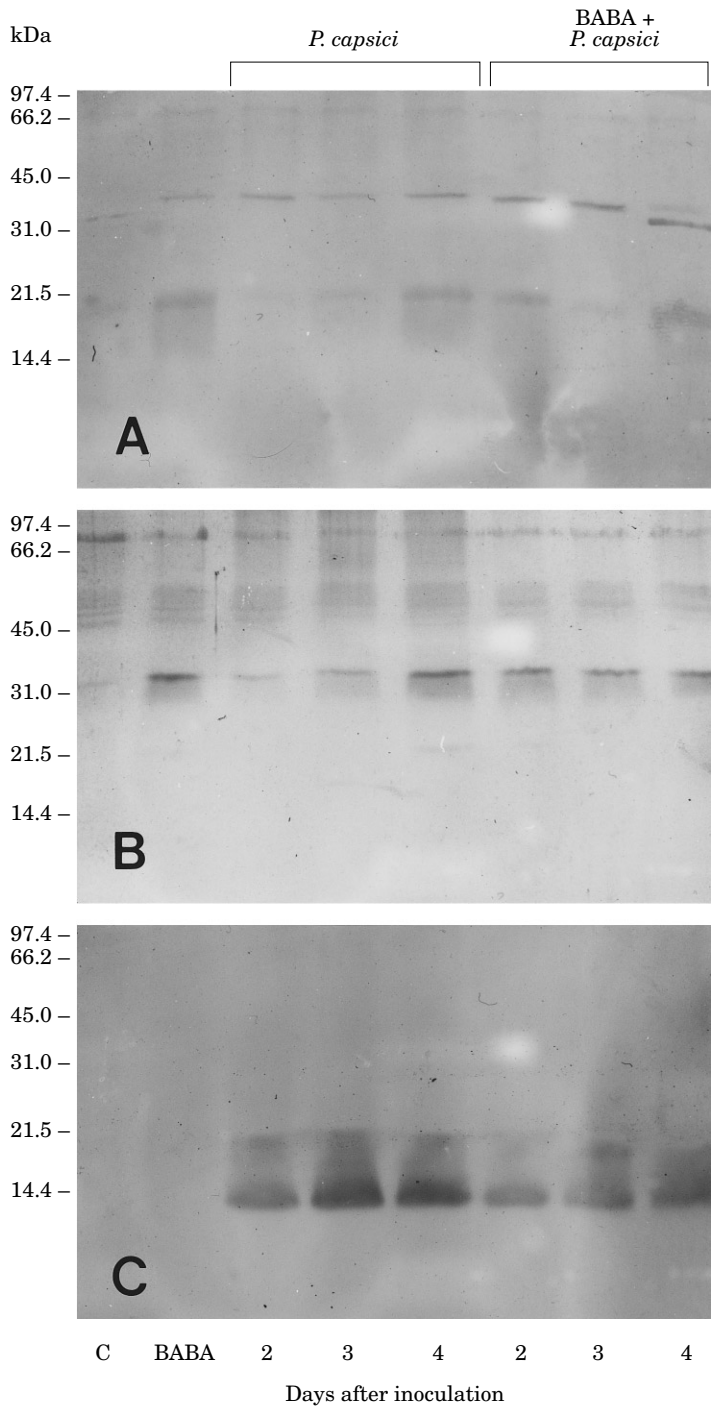

FIG. 6. For legend see opposite.

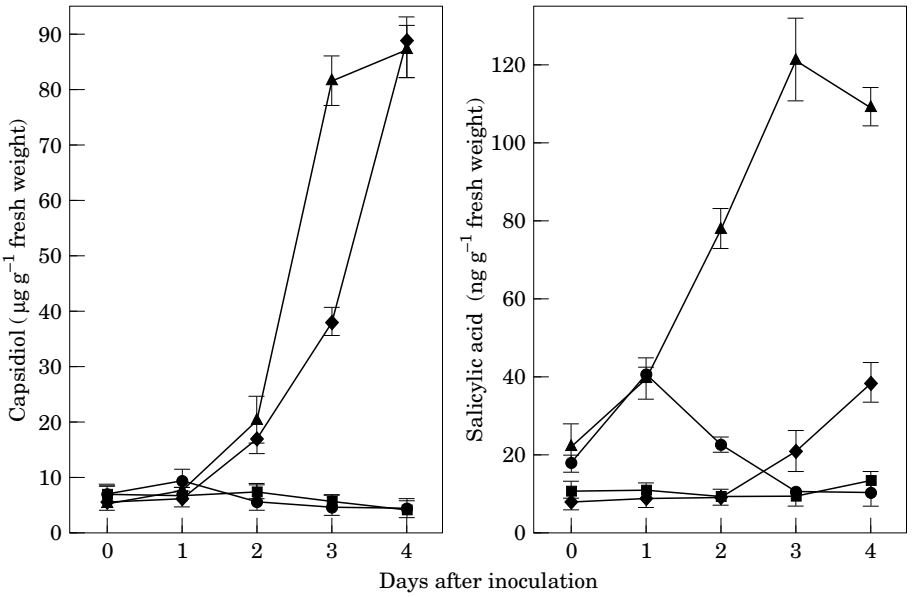

FIG. 7. Levels of capsidiol and salicylic acid in the stem extracts from pepper (cv. Hanbyul) plants at the first-branch stage treated with 1000 μg ml^{-1} of DL-β-amino-n-butyric acid (BABA) and/or challenged 4 days later with *P. capsici*. Vertical bars represent a mean±standard deviations. (■) Untreated; (●) BABA; (◆) *P. capsici*; (▲) BABA+*P. capsici*.

8·8, 9·0, 9·2, 9·3 and 9·4) chitinase isoforms were detected on chitin overlay gel. Four chitinase isoforms with pIs 3·6, 4·1, 4·2, and 9·0 were detected in all the pepper stems tested. Their activity increased markedly in inoculated pepper stems, especially treated with BABA before challenge inoculation. Some basic chitinase isoforms were induced and accumulated only in the pepper stems inoculated with *P. capsici* or ones treated with BABA before challenge inoculation. The basic isoforms (pIs 9·2 and 9·4) were induced only in the infected stems by BABA-treatment.

*Immunodetection of PR proteins in BABA–*P. capsici *interactions*

To examine the induction process of PR proteins further, we analysed western blots of protein extracts from the pepper stems infected with *P. capsici* after treatment with BABA (Fig. 6). Proteins cross-reacting with antisera against tomato β-1,3-glucanase, chitinase, and PR-1 proteins were found in the pepper stems treated with BABA or infected with *P. capsici*. The 32 kDa β-1,3-glucanase isoform was constitutively present in the pepper stems (Fig. 6A). However, the 20 kDa isoform β-1,3-glucanase was induced and accumulated in pepper stems after either BABA treatment or *P. capsici* infection. The 69 kDa chitinase was constitutively expressed in all the pepper stems

FIG. 6. Immunodetection of β-1,3-glucanase (A), chitinase (B), and PR-1 protein (C) in stem extracts from pepper (cv. Hanbyul) plants treated with 1000 μg ml^{-1} DL-β-amino-n-butyric acid and/or 4 days later challenge-inoculated with *P. capsici* at the first-branch stage. Crude enzyme extracts (7 μg protein) were subjected to SDS-PAGE and immunoblotted with antibodies against β-1,3-glucanase, chitinase, or PR-1 proteins of tomato, respectively.

treated (Fig. 6B). In contrast, the 32 kDa chitinase was detected at the low level in the healthy stems, but strongly accumulated in the BABA-treated, *P. capsici* infected or the BABA-treated and infected stems. Figure 6C shows accumulation of proteins in the *P. capsici* infected stems treated with or without BABA, cross-reacting with the antibodies raised against PR-1 protein. The PR-1 homologues were not found in either the healthy untreated or BABA-treated stems. However, the BABA-nontreated stems accumulated more PR-1 proteins after inoculation than BABA-treated and inoculated stems.

Accumulation of capsidiol and salicylic acid related to induced resistance

Concentrations of capsidiol and salicylic acid were measured in the BABA-treated and/or inoculated pepper stems at various times after inoculation with *P. capsici* (Fig. 7). In the pepper stems untreated or treated with BABA, concentrations of capsidiol remained at very low levels. However, capsidiol accumulated strongly in pepper stems inoculated with *P. capsici* after treatment with BABA, especially 3 and 4 days after challenge inoculation. Inoculated, untreated stems also accumulated capsidiol but the accumulation was less marked than in the case of BABA treatments.

The concentration of salicylic acid in stems increased four-fold 5 days after BABA treatment (1 day after challenge inoculation) and gradually declined to the untreated levels (Fig. 7). However, stems inoculated after BABA treatment accumulated more salicylic acid than did inoculated untreated stems with a maximum at 3 days after challenge inoculation.

DISCUSSION

In our recent investigation [*39*] and present experiment (Fig. 1), pepper plants (cv. Hanbyul) treated with the nonprotein amino acid, DL-β-amino-n-butyric acid (BABA) were strongly protected against a challenge inoculation with *P. capsici*. Such findings enabled us to examine whether or not the accumulation of PR proteins such as β-1,3-glucanases and chitinases, the phytoalexin capsidiol, and salicylic acid occur in the induction of resistance to phytophthora blight in pepper plants by treatment with BABA.

Our experiments demonstrated that the treatment with BABA induced the synthesis and accumulation of β-1,3-glucanases and chitinases in the stem tissues of pepper plants (Fig. 2). In particular, accumulation of the two hydrolytic enzymes was remarkably pronounced in pepper stems challenge-inoculated with *P. capsici* after BABA treatment, suggesting a possible stimulation of PR protein accumulation in the infected tissues by BABA treatment. Interestingly, we also found considerable hydrolase increases in pepper stems treated with BABA alone. These results are consistent with the findings of Cohen *et al.* [*7*], which demonstrated that BABA induced the accumulation of high levels of three PR proteins P14a, β-1,3-glucanase, and chitinase in tomato plants. In contrast, Cohen *et al.* [*7*] reported recently that SDS-PAGE analyses did not reveal enhanced levels of PR proteins in BABA-injected tobacco plants.

The use of PAGE and IEF gels [*29*, *41*] could ascertain whether or not the different isoforms of β-1,3-glucanase and chitinase play significant roles in the induction of

resistance to *P. capsici* in pepper plants by BABA treatment. Isoforms Ga 1 of β-1,3-glucanase were induced and accumulated in pepper stems after BABA treatment and their accumulation was markedly enhanced in *P. capsici* infected stems previously treated with BABA (Fig. 3). These data suggest that some isoforms of β-1,3-glucanase may be induced in pepper plants by *P. capsici* infection as well as BABA treatment.

Among the acidic isoforms of chitinase, isoform Ca 2 seemed to be induced and accumulated in pepper stems by either BABA treatment or *P. capsici* infection (Fig. 4). Figure 5 shows that Ca 1 and especially Ca 3 increased significantly more in BABA treated and inoculated stems than in non-treated, inoculated ones. This is consistent with the differences between compatible and incompatible interactions [*19*]. BABA treatment alone did not induce basic chitinase accumulation in pepper stems (Fig. 4). However, the strong induction and accumulation of individual basic chitinase isoforms in BABA-treated and/or *P. capsici*-infected stems may be the basis of the high activity of chitinase found in pepper stems treated with BABA and inoculated with *P. capsici* (Fig. 2). IEF of the stem extracts did not demonstrate the *de novo* synthesis of chitinase isoforms in BABA-treated stems, whereas certain basic isoforms with pIs 9·2 and 9·5 accumulated strongly in stems treated with BABA prior to inoculation with *P. capsici* (Fig. 5). It may well be that BABA induces the accumulation of some basic chitinase isoforms in the pathogenesis response leading to induced resistance in pepper plants.

Immunoblot analysis of the denatured proteins demonstrated the accumulation of β-1,3-glucanase, chitinase, and PR-1 protein in pepper stems after BABA treatment and/or *P. capsici* infection (Fig. 6). The 20 kDa β-1,3-glucanase and 32 kDa chitinase were induced and accumulated in pepper stems not only after BABA treatment, but also after *P. capsici* inoculation. However, PR-1 proteins did not accumulate in either the healthy or the BABA-treated stems. PR-1 proteins accumulated in *P. capsici* infected, non-treated stems stronger than in infected BABA-treated stems, suggesting a possible involvement of the PR protein in the infection process of *P. capsici* rather than in resistance.

Despite the efficacy of BABA in the induction of resistance in pepper plants [*39*], BABA treatment did not stimulate capsidiol production in pepper stems (Fig. 7). However, BABA treatment strongly stimulated capsidiol production in pepper stems infected with *P. capsici*. These results imply that BABA itself does not elicit capsidiol production in pepper stems, but increases the capacity of pepper plants to synthesize capsidiol in response to infection by *P. capsici*. Similar phenomena were well demonstrated by our earlier study of capsidiol production in stems of pepper plants treated with the systemic fungicide metalaxyl and inoculated with *P. capsici* [*13*].

BABA treatment stimulated SA production at least 12-fold in pepper stems by 3 days after inoculation with *P. capsici* (Fig. 7). This increase was much more pronounced than after *P. capsici* infection alone, where SA levels increased two to four-fold only. These results indicate that SA may be an endogenously synthesized compound critical for the induction of resistance to *P. capsici* in pepper plants by BABA. Recently, the only confirmed step in the signal transduction pathway leading to systemic acquired resistance has been shown to be the accumulation of SA [*10*, *24*, *27*]. In contrast, Vernooij *et al*. [*44*] demonstrated recently that resistance to TMV was induced in tobacco by 2,6-dichloroisonicotinic acid without the accumulation of SA.

In conclusion, it seems likely that particular β-1,3-glucanase and chitinase isoforms

may be induced and accumulate in stem tissues in the BABA-induced resistance response to *P. capsici*. Induction of resistance to *P. capsici* in pepper plants by BABA treatment positively correlated with the accumulation of several isoforms of PR proteins such as β-1,3-glucanase and chitinase and endogenous SA in pepper stems. The data presented in this study further support the hypothesis that SA acts as an endogenous signal responsible for activating particular components of resistance to *P. capsici* and the induction of PR proteins in pepper plants [*48*]. More detailed research is needed to elucidate whether or not SA activates additional known and unknown resistance mechanisms in *P. capsici* pepper combinations.

This study was supported in part by special research funds from Korea Ministry of Agriculture and Forestry in 1995–1998.

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