Acibenzolar-S-Methyl-Induced Resistance to Japanese Pear Scab Is Associated with Potentiation of Multiple Defense Responses

Mohamed Faize, Lydia Faize, Nobuyo Koike, Masumi Ishizaka, and Hideo Ishii

National Institute for Agro-Environmental Sciences, 3-1-3 Kannondai, Tsukuba, Ibaraki 305-8604, Japan.
Accepted for publication 12 January 2004.

ABSTRACT

Faize, M., Faize, L., Koike, N., Ishizaka, M., and Ishii, H. 2004. Acibenzolar-S-methyl-induced resistance to Japanese pear scab is associated with potentiation of multiple defense responses. *Phytopathology* 94:604-612.

This study reports the mode of action of acibenzolar-S-methyl (ASM) against Japanese pear scab, caused by *Venturia nashicola*. Pretreatment of potted Japanese pear trees with ASM reduced scab symptoms and potentiated several lines of plant defense response. This included transcripts encoding polygalacturonase-inhibiting protein (PGIP) that were highly and transiently promoted after scab inoculation of plants pretreated with ASM, suggesting a possible role for defenses involved in direct interaction with the pathogen. The activity of the key enzyme of phenylpropanoid pathway, phenylalanine ammonia lyase (PAL), was enhanced in scab-inoculated leaves pretreated with ASM only 7 days after inoculation, suggesting that it may play a minor role in induced resistance. In this

work, salicylic acid (SA) accumulation was enhanced in ASM-treated leaves for the first time, according to an equivalent time course to that of PAL activity. However, a delayed induction of SA accumulation in ASM-treated leaves compared with kinetics of induction of several pathogenesis-related (PR) proteins or their encoding genes suggested that resistance triggered by ASM may be SA-independent. Among these PR proteins, PR-1, chitinase and PR-10 were promoted early by ASM after scab inoculation. Peroxidase, as well as enzymes involved in the oxidative burst such as superoxide dismutase, catalase, and ascorbate peroxidase were weakly activated with ASM treatment alone or pathogen inoculation alone and highly enhanced in ASM pretreated plants upon challenge inoculation, suggesting the occurrence of priming phenomenon during the interaction of Japanese pear-ASM-*V. nashicola*. An early potentiation of the activity of these enzymes after scab inoculation of leaves pretreated with ASM suggested that active oxygen species may be involved as a signal for the activation of PR proteins or genes.

Induced resistance is a plant defense state observed upon infection, conferring a protection against a broad spectrum of pathogens in the area of primary infection and in the distal parts of the plant (23,43). This phenomenon is associated with the accumulation of salicylic acid (SA), required for signal transduction (7), leading to coordinated expression of pathogenesis-related (PR) proteins with antimicrobial activity (49) and lignification (13). Exogenous application of chemicals that mimic natural signaling compounds like SA or its synthetic analogs 2,6-dichloroisonicotinic acid (INA) (36) or acibenzolar-S-methyl (ASM) (27) are also effective in inducing resistance.

Another characteristic of plants expressing resistance is the notion of "priming" of defense responses, which is defined as the ability to induce cellular defense responses more rapidly and effectively to subsequent infection (38). This was first demonstrated on parsley cell cultures after application of elicitors derived from cell walls of *Phytophthora sojae*, resulting in phytoalexin accumulation, oxidative burst (19), and defense gene expression, which are greatly enhanced after pretreatment with SA (48), INA (20), or ASM (18). Subsequent studies revealed this phenomenon on whole plants; Latunde-Dada and Lucas (26) showed that ASM mediated systemic priming of phenylalanine ammonia lyase (PAL), chitinase activity, and phytoalexin accumulation in cowpea seedlings and enhanced resistance against the causal agent of anthracnose, *Colletotrichum destructivum*. In cucumber plants treated with ASM, *pal* gene was systemically primed upon challenge-inoculation with *Colletotrichum orbiculare* (6). Moreover, in Arabidopsis plants, potentiation of defense

mechanisms by β -aminobutyric acid (52) or ASM (21) pretreatment has been shown to be an important cellular mechanism in acquired disease resistance and requires the *nonexpressor of PR1 gene/noninducible immunity 1* gene (21).

Scab, caused by *Venturia nashicola*, is one of the most severe diseases of Japanese pear (*Pyrus pyrifolia* var. *culta*) and Chinese pear (*Pyrus ussuriensis*) (17,47). Damage from this disease is serious because almost all commercially important cultivars are susceptible (15), resulting in high dependence of growers on fungicide use to control this disease. Ishii et al. (14) previously reported the control efficacy of the resistance inducer ASM against Japanese pear scab. Subsequently, field experiments revealed that the degree of control was acceptable in practice only when spray applications were started just after a full blossom of pear trees (16). However, molecular mechanisms of resistance induced by ASM in Japanese pear trees have never been reported.

In the present study, we investigated the mechanism of expression of ASM-mediated resistance in Japanese pear against scab. Several defense responses were analyzed, including transcripts encoding polygalacturonase-inhibiting protein (PGIP), some components of phenylpropanoid pathway, and enzymes involved in the oxidative burst along with PR proteins or their encoding genes.

MATERIALS AND METHODS

Plant material, chemical application, inoculation of the pathogen, and disease assessment. Potted trees of scab-susceptible Japanese pear cv. Kousui were grown in a screenhouse at the National Institute for Agro-Environmental Sciences (Ibaraki, Japan).

Distilled water (DW) or ASM (100 μ g ml⁻¹) (50% wettable granule formulation, a gift from Syngenta Japan) was applied by spraying the young leaves one or two times (7 and 3 days before pathogen inoculation).

Corresponding author: H. Ishii; E-mail address: hideo@niaes.affrc.go.jp

Publication no. P-2004-0406-02R

© 2004 The American Phytopathological Society

Conidia of race 1 of *V. nashicola* collected directly from lesions were suspended in DW, filtered through tissue paper, washed by centrifugation, and resuspended in 0.1% sucrose, and concentrations were adjusted to $\approx 5 \times 10^5$ conidia ml⁻¹. They were sprayed on young, treated leaves of potted trees, and the inoculated plants were incubated in a moist chamber at 20°C for 48 h and transferred to the greenhouse.

For disease assessment, percentage of sporulating leaves, disease severity, and percentage of disease control were recorded 21 and 30 days after inoculation, according to Ishii et al. (14). Comparisons of differences were made using the Student's *t* test, with *P* < 0.05 considered significant.

For molecular and biochemical studies, the inoculated or treated leaves were detached from plants at intervals (0, 1, 2, 3, 5, and 7 days after inoculation) and used immediately or stored at -80°C until use.

Protein extraction and western blot analysis. Proteins were extracted according to the protocol described by Shuster and Davies (44) with slight modifications. One hundred milligrams of leaf tissue (fresh weight) was ground in a mortar and pestle under liquid nitrogen with 0.5 ml of homogenization buffer (0.5 M Tris-HCl [pH 8.7], 0.7 M sucrose, 0.05 M Na₂EDTA, 0.1 M KCl, 14 mM β -mercaptoethanol, and 1 mM phenylmethionine sulfoxide [PMSF]). Five hundred microliters of phenol was added, and the mixture was incubated on ice for 10 min. After centrifugation at $6,000 \times g$ for 10 min, the upper phase was collected. One volume of homogenization buffer was added, and the mixture was centrifuged under the same conditions described previously. The upper phase was then collected and precipitated with 0.1 M ammonium acetate (prepared in methanol) overnight at -20°C. After centrifugation at $10,000 \times g$ for 30 min, the pellet was washed twice with acetone containing 0.07% β -mercaptoethanol, freeze-dried, and dissolved in 50 μ l of Tris-HCl, pH 8.8, containing 5 mM Na₂EDTA and 500 mM sucrose.

Protein concentration was measured by the method of Bradford (3), using bovine serum albumin as a standard and 20 μ g of protein loaded per lane on a gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Laemmli (24). Separated proteins were transferred onto a polyvinylidene difluoride membrane (PVDF Immobilon transfer membrane, pore size 0.45 μ m; Millipore, Bedford, MA). Polyclonal antibody raised against an acidic cucumber chitinase (40), which shows high identity to Japanese pear chitinase, or polyclonal antibody raised against apple PR-10 protein (51) was used separately as a first antibody. Goat anti-rabbit antibody conjugated with horseradish peroxidase (Biomedical Technologies, Stoughton, MA) was used as a secondary antibody. Associated secondary antibodies were detected with an enhanced chemiluminescence (ECL) western blotting detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions.

SA analysis. SA was extracted from scab-inoculated leaves with 10 volumes (vol/wt) of 2% acetic acid. After boiling for 10 min and centrifugation at $2,000 \times g$ for 10 min, extracts were filtered through a Millex-HV filter (0.45 μ m, Millipore), mixed with 1 volume of methanol containing 0.1% of H₃PO₄, and centrifuged again at $16,000 \times g$ for 15 min. Total SA content was determined by high-performance liquid chromatography (Agilent 1100; Agilent Technologies, Palo Alto, CA) using a reverse phase C18 column (Wakosil-II 5 C18 HG column; Wako Pure Chemical, Osaka, Japan) and eluted at 40°C with 50% methanol and 0.05% H₃PO₄ at a flow rate of 1 ml min⁻¹. SA was quantified fluorometrically (290 nm extinction and 402 nm emission). Commercial SA was used as a standard.

Enzyme extraction. Except for ascorbate peroxidase (A-POX, EC 1.11.1.11) and PAL (EC 4.3.1.5) extraction, all other enzymes were extracted by grinding 100 mg of fresh young leaves under liquid nitrogen with 1 ml of 50 mM phosphate buffer, pH 7.5, containing 0.01% (vol/vol) Triton X-100, 1 mM polyethylene

glycol, 1 mM PMSF, and 8% polyvinylpyrrolidone phosphate (PVPP). For A-POX, phosphate buffer was substituted with 50 mM Tris-HCl and 1 mM ascorbate was added to extraction buffer. PAL was extracted in 25 mM Tris-HCl, pH 8.0, containing 1 mM PMSF, 1 mM dithiothreitol, and 2% PVPP. The homogenate was centrifuged at $16,000 \times g$ for 20 min, and the supernatant was assayed immediately for enzymatic activities.

Enzymatic activities. Except for superoxide dismutase (SOD, EC 1.15.1.1), all enzymatic assays were performed with a UV/visible spectrophotometer (Beckman DU 7400; Beckman Instruments, Fullerton, CA). Unless indicated, incubation of the reaction mixture was carried out at room temperature.

SOD activity was assayed by measuring the inhibition of nitroblue tetrazolium (NBT) reduction using the protocol adopted from McCord and Fridovich (34). Microtiter-plate wells were filled with 50 mM phosphate buffer [pH 7.5], 13 mM methionine, 75 μ M NBT, 1 mM EDTA, and 10 μ l of enzyme extract. The reaction was initiated after adding 2 μ M riboflavin and incubating the plates under fluorescent light for 15 min. The absorbance was read at 570 nm with an enzyme-linked immunosorbent assay plate reader (Model 550; Nippon Bio-Rad Laboratories, Tokyo). Controls were unilluminated plates. The volume of extract corresponding to 50% inhibition of the reaction was taken as one enzyme unit (U). Enzyme activity was expressed as one enzyme unit per milligram of protein.

For catalase (CAT, EC 1.11.1.6) activity, a decrease in H₂O₂ absorbance was followed at 240 nm for 2 min (1). The reaction mixture contained 50 mM phosphate buffer [pH 7.5], 25 mM H₂O₂, and 20 μ l of extract. Enzyme activity was expressed as micromoles per minute per milligram of protein ($\epsilon_{240} = 0.04$ mM⁻¹ cm⁻¹).

A-POX activity was followed as a decrease in absorbance at 290 nm during 3 min (39). The reaction mixture contained 50 mM Tris-HCl [pH 7.5], 5 mM ascorbate, and 50 μ l of extract. Adding 0.5 mM H₂O₂ started the reaction, and the rate of decrease was corrected from the nonenzymatic oxidation of ascorbate by measuring the activity without enzyme extract. Enzyme activity was expressed as nanomoles of ascorbate per minute per milligram of protein ($\epsilon_{290} = 2.8$ mM⁻¹ cm⁻¹).

Peroxidase (POX, EC 1.11.1.7) activity was determined at 470 nm as described by Moerschbacher et al. (37). The reaction mixture consisted of 25 mM guaiacol (2-methoxyphenol) prepared in 50 mM phosphate buffer [pH 7.5], 100 mM H₂O₂, and 25 μ l of extract. Changes in absorbance at 470 nm were recorded for 2 min, and enzyme activity was expressed as nanomoles per minute per milligram of protein ($\epsilon_{470} = 26.6$ mM⁻¹ cm⁻¹).

Polyphe nol oxidase (PPO, EC 1.10.3.1) activity was measured as described by Masia et al. (31). The reaction mixture contained 25 mM pyrogallol prepared in 100 mM phosphate buffer, pH 7.5, and 100 μ l of extract. Changes in absorbance were followed for 3 min at 410 nm. The activity of PPO was expressed as arbitrary unit per milligram of protein.

PAL activity was recorded at 290 nm according to Whetten and Sederoff (50). The reaction mixture was 500 μ l of Tris-HCl (pH 8.0), 250 μ l of 10 mM L-phenylalanine (prepared in the same buffer), and 250 μ l of extract. After 1 h of incubation at 37°C, the reaction was stopped with 250 μ l of 6 N HCl. Five hundred microliters of toluene was then added in order to separate trans-cinnamic acid (product of reaction) from L-phenylalanine, which interferes with its absorbance. The mixture was centrifuged at $10,000 \times g$ for 10 min, and the upper phase was recovered and its absorbance was recorded against toluene. For each assay, a blank consisting of the same reaction mixture, which was immediately stopped after addition of L-phenylalanine, was included and values were corrected from endogenous cinnamic acid. Activity was expressed as picokatal per milligram of protein ($\epsilon_{290} = 10^4$ mM⁻¹ cm⁻¹).

RNA extraction, cloning, and expression of defense-related genes. RNA was extracted from young leaves of Japanese pear cv. Kousui under liquid nitrogen with the RNeasy Plant Mini Kit

(Qiagen, Hilden, Germany). After DNase treatment with DNA-free (Ambion, Austin, TX), reverse transcription (RT) was performed according to the manufacturer's instruction using RETRO Script Kit (Ambion). Primers listed in Table 1 were designed from pear sequences listed in GenBank database using the Laser-gene DNA Analysis Software (DNASar, Madison, WI) and used for cDNA cloning. Polymerase chain reaction (PCR) was carried out in a thermal cycler (Amp System 2400; Perkin-Elmer, Norwalk, CT). Cycling parameters were 94°C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min followed by a

TABLE 1. Primer sequences used for gene cloning and reverse transcription-polymerase chain reaction (RT-PCR) experiments

cDNA ^a	Pear primer sequences ^b	Size (bp)	Accession number ^c
CHI	fo 5'-AAAAGTCCTCTCTCGATTG-3' re 5'-TTGTTGGAAGGACTTGTGAC-3'	509	AY338247
PR1	fo 5'-AGTAGGCGTTGGTCCCTT-3' re 5'-GCCAAACACCTGTGTATAA-3'	254	AY338352
PPO	fo 5'-CAAATTCTACAAAGCGATGG-3' re 5'-TGATTGTGGTTTCCTTTGAC-3'	407	AY338251
PGIP	fo 5'-ATCGTTTGGGCGATTTCATTC-3' re 5'-ACTCCCGTAGATCTTATTGTGATT-3'	278	AY333102
EF1 α	fo 5'-ATTGTGGTCATTGGYCAAGT-3' re 5'-CCTATCTTGTAVACATCCTG-3'	709	AY338249

^a CHI = chitinase, PR1 = pathogenesis-related protein 1, PPO = polyphenol oxidase, PGIP = polygalacturonase-inhibiting protein, and EF1 α = elongation factor 1 α .

^b All forward primers were labeled at their 5' end with fluorescein isothiocyanate when used for RT-PCR experiments.

^c Genes cloned and sequenced in this study were registered in GenBank under the indicated accession number.

TABLE 2. Control efficacy of acibenzolar-S-methyl (ASM) against *Venturia nashicola*

Treatment	Sporulating leaves (%)	Control (%)	Disease severity	Control (%)
21 dpi ^a				
ASM ^b	48.3 $\pm$ 5.02 ^c	41.7	33.6 $\pm$ 0.65	40.7
DW	82.9 $\pm$ 12.8	...	56.7 $\pm$ 4.71	...
30 dpi				
ASM	60.3 $\pm$ 11.7	29.9	36.2 $\pm$ 1.27	47.5
DW	86.0 $\pm$ 7.07	...	69.0 $\pm$ 5.31	...

^a Days postinoculation.

^b Distilled water (DW) or ASM (100 μ g ml⁻¹) was applied to Japanese pear leaves two times (7 and 3 days) before inoculation with *V. nashicola*.

^c Values are means of three replicates $\pm$ confidence interval (95%); each of the four ASM means is significantly different from its corresponding DW control.

final extension at 72°C for 15 min. The cDNA products were purified and subcloned in pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's protocol. Plasmid DNA was purified by the Wizard Plus SV Minipreps DNA Purification System (Promega). Subcloned products were sequenced by the dideoxynucleotide method with the BigDye Terminator version 3.0 (Applied Biosystems, Foster City, CA) from both strands according to the manufacturer's instructions. The sequence data were registered in GenBank under the accession numbers indicated in Table 1. Gene expression was appreciated on inoculated leaves by a fluorescent-based semiquantitative RT-PCR using forward primers labeled with fluorophore, fluorescein isothiocyanate at their 5' ends. PCR conditions were the same as above except that only 25 cycles, which were within the logarithmic range of amplification, were performed. Amplification of the translation elongation factor 1 α (*efl*- α) (30) was used to adjust initial amounts of RT products from each sample in each PCR reaction. These primers, previously designed to space an intron sequence (42), allowed us to check for absence of genomic DNA contamination. First, RT-PCR was carried out with EF1- α primers. In a second reaction, comparative amplification was performed with other primers on equivalent total cDNA. Amplification products of studied genes and *efl*- α cDNA were separated in an agarose gel (1.2%), visualized, and analyzed using Bio-Rad's FX Molecular Bioimager and Quantity One Quantitation Software (Nippon Bio-Rad, Tokyo). The ratio of expression between target gene and *efl*- α was determined and plotted as fold increases above values measured at 0 days (immediately after treatment and after checking that this value remained unchanged from that recorded in noninoculated leaves).

RESULTS

Control efficacy of ASM on Japanese pear scab. Under severe disease pressure, two applications of ASM at 100 μ g ml⁻¹ at 7 and 3 days before inoculation with *V. nashicola* induced a significant reduction in the percentage of diseased leaves, as well as in the disease severity compared with that of DW treatments (Table 2). When sporulating leaves were recorded, the control efficacy of ASM decreased from 42% at 3 weeks after inoculation to 30% at 1 month after inoculation. However, the control efficacy was not affected when disease severity was used for disease assessment.

Expression of *pgip* gene involved in the inactivation of pathogenicity factors. PGIPs are involved in the inactivation of pathogen polygalacturonases (8). To assess their role in ASM-mediated defense against Japanese pear scab, semiquantitative RT-PCR was used to follow the expression of 278-bp *pgip* cDNA on RNA extracted from leaves pretreated two times with ASM or DW and harvested after scab inoculation (Fig. 1). A weak activa-

Fig. 1. Semiquantitative reverse transcription-polymerase chain reaction analysis of polygalacturonase-inhibiting protein (*pgip*) gene expression in young Japanese pear leaves inoculated with *Venturia nashicola*, pretreated two times with acibenzolar-S-methyl (ASM) (100 μ g ml⁻¹) or distilled water (DW). The size of the amplicon of *pgip* gene transcripts was 278 bp and the size of elongation factor 1 α (*efl*- α) gene transcripts was 709 bp. The latter was used as a constitutive control. Gel shown is representative of three independent replicates tested per treatment. The ratio of *pgip*/*efl*- α was determined and plotted as fold increases above values measured at 0 days. Values represent the mean $\pm$ confidence interval (95%).

tion of the *pgip* gene was noticed early (2 days after inoculation) in leaves pretreated with DW, whereas a high and transient activation was observed in leaves pretreated with ASM and was 2.5 times higher than leaves in the control.

Activity of enzymes involved in the oxidative burst. The activity of three enzymes (SOD, CAT, and A-POX) involved in the oxidative burst was compared among potted trees pretreated two times with ASM or DW and inoculated with *V. nashicola*. Sucrose solution was used as a control. These experiments were carried out several times during 2002 and 2003, and data of typical results are presented.

The time course of SOD activity is shown in Figure 2A. When compared with the control (DW treatment/sucrose treatment), ASM induced a significant increase in SOD activity only at 2 and 5 days after treatment with sucrose. In response to scab challenge, a significant increase was recorded in leaves treated with DW at 5 days after inoculation. Such an increase tended to be higher in leaves treated with ASM. In this case, SOD activity increased even on the day of scab inoculation and remained significantly higher until the second day. At 3 days after inoculation, SOD activity increased in the control; this was observed in several independent experiments and was perhaps due to our experimental conditions (transferring plants from the moist chamber to the greenhouse).

For CAT activity (Fig. 2B), when compared with the control, only a small activation was induced with ASM at 2 and 7 days after treatment with sucrose. After scab challenge, no activation was noticed in leaves pretreated with DW during the 7 days of the experiment, whereas a higher activation was recorded in leaves pretreated with ASM even on the day of inoculation. This significant activation reached its maximum at 2 days after scab inoculation and remained significantly higher until the third day.

A-POX (Fig. 2C) was significantly activated with ASM at 2 and 3 days after sucrose treatment compared with the control (DW). In response to scab challenge, the activation observed with ASM, at these times, was highly promoted.

Expression of PPO, PR proteins, and their encoding genes. The expression of PPO as well as several PR proteins and/or their encoding genes was studied with potted trees pretreated with ASM or DW and inoculated. These included POX, CHI, PR-1, and PR-10.

The time course of PPO induction was compared in leaves pretreated one or two times with ASM or DW after scab challenge (Fig. 3A). Although an increase in PPO activity was recorded in leaves pretreated two times with ASM from 2 days after scab challenge until the seventh day of the experiment, it was not significantly different from that observed in DW or one ASM treatment.

In order to confirm data of enzymatic activities, *ppo* gene expression was studied by RT-PCR analyses with RNA extracted from leaves pretreated two times with ASM or DW prior to scab inoculation (Fig. 3B). No difference in signal intensity could be found between ASM- and DW-treated leaves.

The time course of POX induction was first analyzed in leaves pretreated one or two times with ASM prior to scab challenge (Fig. 4A). When compared with DW, a small increase was recorded in leaves treated one time with ASM from 1 day after inoculation until the seventh day. In leaves treated two times with ASM, this increase was significantly higher (four to six times) than in the control. These experiments were repeated three times in 2002, with similar results.

In 2003, other experiments were carried out using leaves treated two times with ASM or DW and treated with sucrose or *V. nashicola* (Fig. 4B). In leaves treated with ASM and treated with sucrose, a slight but significant increase was observed at 1 and 3 days after inoculation compared with leaves treated with DW and treated with sucrose or *V. nashicola*. However, this increase was lower than that observed in leaves treated with ASM

and scab-inoculated. In this case, POX activity was significantly enhanced even at 5 and 7 days after inoculation.

The expression of PR-10 polypeptide was followed in scab-challenged leaves after two treatments with ASM or DW by western blot analysis using an antibody raised against apple PR-10 (Fig. 5). A faint 18-kDa band, which matched the molecular mass of PR-10, was detected in protein extract from leaves pretreated with DW, after challenge inoculation. In protein samples from ASM-treated leaves, the intensity of this band was denser even on the day of scab inoculation and it reached its maximum of intensity 3 days after inoculation and then decreased afterward. This experiment was carried out three times (two times in 2002 and

Fig. 2. Time course of changes in **A**, superoxide dismutase activity, **B**, catalase activity, and **C**, ascorbate peroxidase activity in inoculated Japanese pear leaves. Leaves were sprayed two times with acibenzolar-S-methyl (ASM) at 7 and 3 days before treatment with sucrose or inoculation with spore suspensions of *Venturia nashicola* prepared in sucrose solution. Values represent the mean of four replicates $\pm$ confidence interval (95%).

one time in 2003) with similar results. In leaves treated with DW or ASM and treated with sucrose, a very weak band was only occasionally detected (data not shown).

The expression of CHI was also followed in scab-challenged leaves after two treatments with ASM or DW by western blot analysis using an antibody raised against an acidic cucumber CHI (Fig. 6A). A 30-kDa band was detected in the control at 0 days after scab inoculation and its intensity increased between 2 and 3 days after inoculation and then tended to decrease in the following days. In leaves treated two times with ASM, this protein showed denser intensity 1 day after scab inoculation and remained higher during the whole duration of the experiment. This experiment was carried out two times in 2002, and similar results were obtained.

In order to confirm data of western blot analyses, the expression of *chi* gene was further analyzed by RT-PCR using RNA extracted from pear leaves treated two times with ASM or DW and scab-inoculated (Fig. 6B). A transient activation (1 and 2 days after inoculation) was noticed in both inoculated leaves and it was more pronounced in ASM-treated leaves than in the leaves treated with DW. This experiment was conducted four times with similar results, indicating a positive correlation of CHI between gene expression and protein accumulation.

In the same conditions, *pr-1* gene expression also was analyzed (Fig. 7). Similar kinetics of induction were observed for the *chi* gene; however, difference between ASM and DW treatments was more pronounced for *pr-1* than for *chi* even on the day of inoculation (sixfold increase).

PAL activity and SA accumulation. To assess the role of phenylpropanoid pathway in scab resistance induced by ASM, the time course of PAL activity and SA content were analyzed in

scab-inoculated leaves of Japanese pear after two treatments with ASM or DW.

In nontreated and noninoculated leaves, the basal activity of PAL was about 3.44 ± 0.42 pkat per mg of proteins (data not shown). In leaves treated with DW and then scab-inoculated, no significant change in PAL activity was recorded (Fig. 8A). In leaves treated with ASM, a significant increase was observed only 7 days after inoculation.

No significant difference in SA accumulation was observed between leaves treated with DW or ASM after sucrose treatment (data not shown). However, after scab challenge, SA accumulated at significantly higher levels in leaves treated with ASM than in leaves treated with DW at 7 days after inoculation (Fig. 8B). This experiment was carried out in 2002 and 2003 with similar tendency.

DISCUSSION

ASM has been shown previously to be effective in reducing scab disease of Japanese pear caused by *V. nashicola* in the field, despite the fact that no antifungal activity was observed (14,16). In this paper, we investigated mechanisms of this protection. Defense responses of pear are poorly studied and mechanisms of resistance in the tripartite interaction between Japanese pear–ASM–*V. nashicola* have never been reported. Our results indicated that pretreatment of potted Japanese pear trees with ASM at $100 \mu\text{g ml}^{-1}$ two times before challenge inoculation enhanced resistance against scab. These results are also consistent with the report by Brisset et al. (4) who showed that high control of fire blight on apple trees was achieved after several ASM treatments. This was correlated with the direct effect of ASM to activate POX and

Fig. 3. Time course of changes in **A**, polyphenol oxidase (PPO) activity and **B**, *ppo* gene expression in Japanese pear leaves inoculated with *Venturia nashicola*. Values represent the mean of six replicates (from three separate experiments) $\pm$ confidence interval (95%). *ppo* gene expression was analyzed by reverse transcription-polymerase chain reaction on inoculated plants after two treatments with acibenzolar-S-methyl (ASM) or distilled water (DW). The size of the amplicon of *ppo* gene transcripts was 407 bp and the size of elongation factor 1 α (*efl-1*) gene transcripts was 709 bp. The latter was used as a constitutive control. Gel shown is representative of three independent replicates tested per treatment. The ratio of *ppoefl-1* was determined and plotted as fold increases above values measured at 0 days. Values represent the mean $\pm$ confidence interval (95%).

glucanases (4,33). However, it is also possible that, after ASM treatment, the plant becomes sensitized in such a manner that its defense response to a subsequent pathogen attack is produced earlier and/or more intensively. To assess this second hypothesis, several genes and/or their encoding proteins were analyzed in ASM pretreated trees after challenge inoculation. In most of the studies dealing with the effect of resistance inducers, only a few defense markers were monitored. In this paper, we focused on additional lines of defense response, which have never been monitored after ASM treatment; this included PGIP. The receptor-like leucine-rich molecular structure of PGIP provides a molecular basis for its putative role as a secreted receptor component of the cell wall signaling system involved in recognition events between plant and fungi (8). PGIP are involved in the inactivation of fun-

gal polygalacturonases, and have been demonstrated to be activated in response to wounding, or treatments with SA and fungal infection (2,29,41). In Japanese pear, *pgip* gene expression was induced rapidly after inoculation with *V. nashicola* and was highly promoted by ASM treatment in inoculated leaves, reinforcing the hypothesis that PGIP functions in resistance of plants to fungal attack. In addition, it has been suggested that the interaction between PGIP and polygalacturonases may favor the accumulation of elicitor-active fractions resulting in the activation of host defense response (5).

An early line of plant defense response is the oxidative burst, which involves a rapid generation of active oxygen species (AOS), such as $O_2^{\cdot-}$ or H_2O_2 , known to orchestrate several lines of active defense (9). In this study, generation of AOS was also compared in inoculated leaves pretreated with ASM or DW, indirectly through the activation of three antioxidative enzymes: SOD, CAT, and A-POX. The activity of SOD, which is involved in $O_2^{\cdot-}$ dismutation, was already enhanced in leaves treated with ASM on the day of scab inoculation (7 days after the first ASM treatment). In the same leaves, a transient increase was observed for CAT and A-POX. These enzymes were weakly activated with ASM treatment alone or pathogen inoculation alone and highly enhanced in ASM pretreated plant upon challenge inoculation, suggesting the occurrence of a priming phenomenon in Japanese pear. Interestingly, time of enhancement of these enzymes (2 days after inoculation) coincided with early infection behavior of the pathogen, suggesting an effective role of these enzymes in ASM-mediated resistance.

The expression of resistance is often accompanied by the activation of phenol-oxidizing enzymes such as POX and PPO (11). Increase in PPO activity may contribute to defense through the production of oxidized forms of quinones, which can inactivate pectinolytic enzymes produced by pathogens (28). In this work, however, neither PPO activity nor its encoding gene was enhanced by ASM treatment. In contrast, POX activity was rapidly and highly enhanced in inoculated leaves pretreated with ASM. This suggests that POX could play an effective role in the observed resistance. POX has been associated with induced resistance (13) and involved in several plant defense mechanisms, such as lignin biosynthesis, oxidative cross-linking of plant cell walls, and also generation of AOS (22,25,35). In this report, we showed that POX was slightly induced after inoculation with *V. nashicola* or ASM treatment, and it was further potentiated after at least two ASM treatments. This result is, in part, consistent with that of Cools and Ishii (6) who showed that in cucumber this enzyme was directly induced by ASM but its expression was further enhanced upon elicitation with a fungal pathogen. As for disease reduction, two pretreatments with ASM were more effective than a single treatment (data not shown), for the activation of, at least, three enzymes studied (SOD, CAT, and POX). Thus, disease resistance and the activity of these markers are positively correlated.

Fig. 4. Time course of changes in peroxidase activity in inoculated Japanese pear leaves. **A**, Experiments carried out in 2002 on plants inoculated with *Venturia nashicola* after treatment with distilled water (DW), one acibenzolar-S-methyl (ASM) treatment, or two ASM treatments. Values are the mean of six replicates $\pm$ confidence interval (95%). **B**, Experiments carried out in 2003 and treatments are as follows: DW plus sucrose, ASM plus sucrose, DW plus *V. nashicola*, or ASM plus *V. nashicola*. Values represent the mean of three replicates $\pm$ confidence interval (95%).

Fig. 5. Time course of changes in pathogenesis-related protein 10 (PR-10) accumulation in Japanese pear leaves inoculated with *Venturia nashicola* after two treatments with acibenzolar-S-methyl (ASM) or distilled water (DW). Crude proteins (20 μ g per lane) from inoculated leaves were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and PR-10 was detected by western blotting with a polyclonal antibody raised against apple PR-10. The expected 18-kDa band is indicated by an arrow. Gel shown is representative of three independent replicates tested per treatment.

The expression of PR genes or proteins has been correlated with resistance in many plants and recently in apple seedlings (4,33,51). ASM has been shown to induce their expression; consequently, it is not surprising that ASM triggers these genes in Japanese pear. Our results indicated that PR-1, CHI, and PR-10 could play a role in the observed resistance because their expression was rapidly enhanced in ASM pretreated plants upon challenge inoculation compared with that of DW pretreated leaves.

PAL is a key enzyme of phenylpropanoid pathway that leads to a variety of defense-related plant secondary metabolites such as SA, phytoalexins, and lignin-like polymers (12). It has been shown to play a critical role in ASM-mediated resistance, as its expression was primed early by ASM in Arabidopsis (21), cowpea (26), and cucumber (6). However, the direct role of PAL in resistance induced by ASM comes from the work of Standik and Buchenauer (46) who showed that chemical inhibition of PAL

Fig. 6. Time course of changes in **A**, chitinase (CHI) protein accumulation and **B**, *chi* gene expression in Japanese pear leaves inoculated with *Venturia nashicola* after two treatments with acibenzolar-*S*-methyl (ASM) or distilled water (DW). For CHI protein accumulation, crude proteins (20 µg per lane) from inoculated leaves were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and CHI was detected by western blotting with a polyclonal antibody raised against an acidic cucumber CHI. The expected 30-kDa band is indicated by an arrow and the gel shown is representative of two independent replicates tested per treatment. *chi* gene expression was analyzed by reverse transcription-polymerase chain reaction. The size of the amplicon of *chi* gene transcripts was 509 bp and the size of elongation factor 1α (*efl-α*) gene transcripts was 709 bp. The latter was used as a constitutive control. Gel shown is representative of four independent replicates tested per treatment. The ratio of *chi/efl-α* was determined and plotted as fold increases above values measured at 0 days. Values represent the mean ± confidence interval (95%).

Fig. 7. Semiquantitative reverse transcription-polymerase chain reaction analysis of pathogenesis-related protein 1 (*pr1*) gene expression in Japanese pear leaves inoculated with *Venturia nashicola*, pretreated two times with acibenzolar-*S*-methyl (ASM) or distilled water (DW). The size of the amplicon of *pr1* gene transcripts was 254 bp and the size of elongation factor 1α (*efl-α*) gene transcripts was 709 bp. The latter was used as a constitutive control. Gel shown is representative of four independent replicates tested per treatment. The ratio of *pr1/efl-α* was determined and plotted as fold increases above values measured at 0 days. Values represent the mean ± confidence interval (95%).

abolished resistance in wheat induced against *Blumeria graminis* f. sp. *tritici*. In Japanese pear, ASM is effective in promoting PAL activity. However, a delayed induction of this enzyme (7 days after scab inoculation) may raise the question of its role in resistance against *V. nashicola*. Our results of SA accumulation showed a similar pattern with PAL activity. In Japanese pear leaves treated with ASM and inoculated with scab, SA content was enhanced but only at 7 days after inoculation. However, to our knowledge, these results demonstrate for the first time that ASM potentiates SA accumulation in inoculated Japanese pear while it is well known that ASM alone did not cause any SA accumulation (10). The positive correlation between induced PAL activity and SA accumulation is not surprising because PAL catalyzes the first biosynthetic step in phenylpropanoid pathway leading to SA accumulation (32,45).

SA has been shown to be an intermediary signal leading to the production of PR proteins during systemic acquired resistance (43). As shown in this work, all of the PR proteins or genes analyzed were promoted before SA accumulation, indicating that their induction may be SA-independent. The early activation of enzymes involved in the oxidative burst may suggest a possible role of AOS as a signal leading to PR protein promotion.

In conclusion, the data presented in this paper indicated that protection afforded by ASM in Japanese pear against scab disease was correlated with the enhancement of multiple defense re-

sponses during infection. This wide variety of defense included not only the classic molecular markers for induced resistance, such as PR proteins, but also others involved in the oxidative burst and in direct interaction with pathogens, and some components of the phenylpropanoid pathway.

ACKNOWLEDGMENTS

M. Faize was supported by the Japanese Society for Promotion of Sciences (JSPS). We thank P. Poupard, University of Angers, France, for supplying the antibody anti-PR-10; R. Ishikawa, Takeda Chemical Industries, Ltd., for technical guidance; and the National Institute of Fruit Tree Science, Tottori Horticultural Experiment Station, and Akita Fruit Tree Experiment Station for providing pear scions.

LITERATURE CITED

1. Aebi, H. 1974. Catalases. Pages 673-684 in: Methods of Enzymatic Analysis. H. U. Bergmeyer, ed. Academic Press, New York.
2. Bergmann, C. W., Ito, Y., Singer, D., Albersheim, P., Darwill, A. G., Benhamou, N., Nuss, L., Salvi, G., Cervone, F., and De Lorenzo, G. 1994. Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. *Plant J.* 5:625-634.
3. Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
4. Brisset, M. N., Cesbron, S., and Paulin, J. P. 2000. Acibenzolar-S-methyl induces the accumulation of defense-related enzymes in apple and protects from fire blight. *Eur. J. Plant Pathol.* 106:529-536.
5. Cervone, F., Hahn, M. G., De Lorenzo, G., Darwill, A., and Albersheim, P. 1989. Host pathogen interactions. XXXIII. A plant protection converts a fungal pathogenesis factor into an elicitor of plant defense responses. *Plant Physiol.* 90:542-548.
6. Cools, H. J., and Ishii, H. 2002. Pre-treatment of cucumber plants with acibenzolar-S-methyl systemically primes a phenylalanine ammonia lyase gene (PAL) for enhanced expression upon fungal pathogen attack. *Physiol. Mol. Plant Pathol.* 61:273-280.
7. Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. 1994. A central role for salicylic acid in plant disease resistance. *Science* 266:1247-1250.
8. De Lorenzo, G., D'Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* 39:313-335.
9. Doke, N., Park, H. J., Katou, S., Komatsubara, H., Makino, T., Ban, S., Sugie, K., Yoshioka, H., and Kawakita, K. 2001. The oxidative burst in plants: Mechanisms and function in induced resistance. Pages 184-193 in: Delivery and Perception of Pathogen Signals in Plants. N. T. Keen, S. Mayama, J. E. Leach, and S. Tsuyumu, eds. The American Phytopathological Society, St. Paul, MN.
10. Friedrich, L., Lawton, K., Ruess, W., Masner, P., Speicher, N., Gut-Rella, M., Meier, B., Dincher, S., Staub, T., Uknes, S., Métraux, J. P., and Ryals, J. 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J.* 10:61-70.
11. Goodman, R. N., and Novacky, A. J. 1994. The bacteria-induced hypersensitive reaction. Pages 117-173 in: The Hypersensitive Reaction in Plants to Pathogens. The American Phytopathological Society, St. Paul, MN.
12. Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40:347-369.
13. Hammerschmidt, R., Nuckles, E. M., and Kuć, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Plant Pathol.* 20:73-82.
14. Ishii, H., Tomita, Y., Horio, T., Narusaka, Y., Nishimura, K., and Iwamoto, S. 1999. Induced resistance of acibenzolar-S-methyl (CGA 245704) to cucumber and Japanese pear diseases. *Eur. J. Plant Pathol.* 105:77-85.
15. Ishii, H., Udagawa, H., Nishimoto, S., Tsuda, T., and Nakashima, H. 1992. Scab resistance in pear species and cultivars. *Acta Phytopathol. Entomol. Hung.* 27:293-298.
16. Ishii, H., Watanabe, H., and Tanabe, K. 2002. *Venturia nashicola*: Pathological specialization on pears and control trials with resistance inducers. *Acta Hort.* 587:613-621.
17. Ishii, H., and Yanase, H. 2000. *Venturia nashicola*, the scab fungus of Japanese and Chinese pears: A species distinct from *V. pirina*. *Mycol. Res.* 104:755-759.
18. Katz, V. A., Thulke, O. U., and Conrath, U. 1998. A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. *Plant Physiol.* 117:1333-1339.

Fig. 8. Time course of changes in **A**, phenylalanine ammonia lyase (PAL) activity and **B**, salicylic acid (SA) accumulation in Japanese pear leaves inoculated with *Venturia nashicola* after two treatments with acibenzolar-S-methyl (ASM) or distilled water (DW). For PAL activity, values represent the mean of four to six replicates $\pm$ confidence interval (95%). For SA, accumulation data from the representative experiment are shown.

19. Kauss, H., and Jeblick, W. 1995. Pretreatment of parsley suspension cultures with salicylic acid enhances spontaneous and elicited production of H₂O₂. *Plant Physiol.* 108:1171-1178.
20. Kauss, H., Theisinger-Hinkel, E., Mindernmann, R., and Conrath, U. 1992. Dichloroisonicotinic and salicylic acid, inducers of systemic acquired resistance, enhance fungal elicitor responses in parsley cells. *Plant J.* 2:665-660.
21. Kohler, A., Schwindling, S., and Conrath, U. 2002. Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPRI/NIM1* gene in *Arabidopsis*. *Plant Physiol.* 128:1046-1056.
22. Kombrink, E., and Somssich, E. 1995. Defense responses of plants to pathogens. *Adv. Bot. Res.* 21:1-34.
23. Kuć, J. 1995. Systemic induced resistance. *Asp. Appl. Biol.* 42:235-242.
24. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* 227:680-685.
25. Lamb, C., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:251-275.
26. Latunde-Dada, A. O., and Lucas, J. A. 2001. The plant defense activator acibenzolar-S-methyl primes cowpea [*Vigna unguiculata* (L.) Walp.] seedlings for rapid induction of resistance. *Physiol. Mol. Plant Pathol.* 58:199-208.
27. Lawton, K. A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., and Ryals, J. 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10:71-82.
28. Leatham, G. F., King, M., and Stahmann, M. A. 1980. In vitro protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. *Phytopathology* 70:1134-1140.
29. Machinandiarena, M. F., Olivieri, F. P., Daleo, G. R., and Oliva, C. R. 2001. Isolation and characterization of a polygalacturonase-inhibiting protein from potato leaves. Accumulation in response to salicylic acid, wounding and infection. *Plant Physiol. Biochem.* 39:129-136.
30. Mahé, A., Grisvard, J., and Dron, M. 1992. Fungal and specific gene markers to follow the bean-anthrax infection process and normalize the bean chitinase mRNA induction. *Mol. Plant-Microbe Interact.* 5:242-248.
31. Masia, A., Ventura, M., Gemma, H., and Sansavini, S. 1998. Effect of some plant growth regulator treatments on apple fruit ripening. *Plant Growth Regul.* 25:127-134.
32. Mauch-Mani, B., and Slusarenko, A. J. 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell* 8:203-212.
33. Maxson-Stein, K., He, S.-Y., Hammerschmidt, R., and Jones, A. L. 2002. Effect of treating apple trees with acibenzolar-S-methyl on fire blight and expression of pathogenesis-related protein genes. *Plant Dis.* 86:785-790.
34. McCord, J. M., and Fridovich, I. 1969. Superoxide dismutase: An enzymic function for erythrocyte peroxidase (hemocuprein). *J. Biol. Chem.* 244:6049-6055.
35. Mehdy, M. C. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiol.* 105:467-472.
36. Métraux, J. P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. 1991. Induced resistance in cucumber in response to 2, 6-dichloroisonicotinic acid and pathogens. Pages 432-439 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
37. Moerschbacher, B., Heck, B., Kogel, K. H., Obst, O., and Reisener, H. G. 1986. An elicitor of the hypersensitive lignification response in wheat leaves isolated from the rust fungus *Puccinia graminis* f. sp. *tritici*. II. Induction of enzymes correlated with the biosynthesis of lignin. *Z. Naturforsch.* 41c:839-844.
38. Mur, L. A., Naylor, G., Warner, S. A. J., Sugars, J. M., White, R. F., and Draper, J. 1996. Salicylic acid potentiates defense gene expression in tissue exhibiting acquired resistance to pathogen attack. *Plant J.* 9:559-571.
39. Nakano, Y., and Asada, K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* 22:867-880.
40. Narusaka, Y., Narusaka, M., Horio, T., and Ishii, H. 1999. Induction of disease resistance in cucumber by acibenzolar-S-methyl and expression of resistance-related genes. *Ann. Phytopathol. Soc. Jpn.* 65:116-122.
41. Nuss, L., Mahe, A., Clark, A. J., Grisvard, J., Dron, M., Cervone, F., and De Lorenzo, G. 1996. Differential accumulation of PGIP (polygalacturonase-inhibiting protein) mRNA in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum*. *Physiol. Mol. Plant Pathol.* 48:83-89.
42. Rosati, C., Cadic, A., Duron, M., Renou, J. P., and Simoneau, P. 1997. Molecular cloning and expression analysis of dihydroflavonol 4-reductase gene in flower organs of *Forsythia × intermedia*. *Plant Mol. Biol.* 15:303-311.
43. Ryals, J. A., Neuenschwander, K. H., Willits, M. G., Molina, A., Steiner, H. Y., and Hunt, M. D. 1996. Systemic acquired resistance. *Plant Cell* 8:1809-1819.
44. Shuster, A. M., and Davies, E. 1983. Ribonucleic acid and protein metabolism in pea hypocotyl. I. The aging process. *Plant Physiol.* 73:809-813.
45. Smith-Becker, J., Marois, E., Huguet, E. J., Midland, S. L., Sims, J. J., and Keen, N. T. 1998. Accumulation of salicylic acid and 4-hydroxybenzoic acid in phloem fluids of cucumber during systemic acquired resistance is preceded by a transient increase in phenylalanine ammonia-lyase activity in petioles and stems. *Plant Physiol.* 116:231-238.
46. Standik, M. J., and Buchenauer, H. 2000. Inhibition of phenylalanine ammonia-lyase suppresses the resistance induced by benzothiadiazole in wheat to *Blumeria graminis* f. sp. *tritici*. *Physiol. Mol. Plant Pathol.* 57:25-34.
47. Tanaka, S., and Yamamoto, S. 1964. Studies on pear scab. II. Taxonomy of the causal fungus of Japanese pear scab. *Ann. Phytopathol. Soc. Jpn.* 29:128-136.
48. Thulke, O. U., and Conrath, U. 1998. Salicylic acid has a dual role in the activation of defense-related genes in parsley. *Plant J.* 14:35-43.
49. Van Loon, L. C., and van Strien, E. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55:85-97.
50. Whetten, R. W., and Sederoff, R. R. 1992. Phenylalanine ammonia-lyase from loblolly pine. *Plant Physiol.* 98:380-386.
51. Ziadi, S., Poupard, P., Brisset, M. N., Paulin, J. P., and Simoneau, P. 2001. Characterization in apple leaves of two subclasses of PR-10 transcripts inducible by acibenzolar-S-methyl, a functional analogue of salicylic acid. *Physiol. Mol. Plant Pathol.* 59:33-43.
52. Zimmerli, L., Jakab, G., Métraux, J. P., and Mauch-Mani, B. 2000. Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β -aminobutyric acid. *Proc. Natl. Acad. Sci. USA* 97:12920-12925.