Eur J Plant Pathol (2011) 129:371–378
DOI 10.1007/s10658-010-9687-9

2-Aminobenzoic acid of *Bacillus* sp. BS107 as an ISR determinant against *Pectobacterium carotovorum* subsp. *carotovotrum* SCC1 in tobacco

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Accepted: 8 September 2010 / Published online: 11 December 2010

Abstract Root drench-treatment of tobacco seedlings with *Bacillus* sp. BS107 (BS107) suppressed disease development caused by the pathogen *Pectobacterium carotovorum* subsp. *carotovotrum* SCC1 (SCC1). A determinant of BS107 involved in induced systemic resistance (ISR) against SCC1 was isolated from cell-free culture. ISR bioassay-guided isolation was involved in determining active fractions during chromatography. Mass spectrometry and NMR analyses of the isolated metabolite identified 2-aminobenzoic acid (2-AB) as a main ISR determinant. 2-AB at 2.3 mM suppressed significantly disease development and exhibited no direct contact inhibition of pathogen. Reverse Transcriptase (RT)-PCR analyses of tobacco leaves revealed up-regulation of the induced resistance marker genes such as *PR1a*, *PR1c*, *PR2* and *PR4* by application of 2-AB on the root. Among aminobenzoic acids tested, 2- and 4-aminobenzoic acids showed ISR activity against soft-rot pathogen, but 3-aminobenzoic acid did not. This is the first report that 2-AB exhibits the ISR against SCC1in tobacco. BS107 can play a role in promoting plant defences by secretion of bacterial determinants including 2-AB for elicitation of ISR.

Keywords Aminobenzoic acid · *Bacillus* sp. BS107 · Induced systemic resistance · Soft-rot disease

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Introduction

It is well known that plant growth-promoting rhizobacteria (PGPR) help plants to induce resistance against pathogens by secreting biologically-active compounds. Due to their immobility, plants should develop efficient defence mechanisms against plant pathogens, such as fungi, bacteria, and viruses and plant insects and nematodes, by interacting with beneficial rhizobacteria in the soil environment (Cui et al. 2005). Treatment with PGPR around plant roots enhances plant defence systems, providing an environmentally friendly mean as an alternative to synthetic chemicals. Among induction of plant defence mechanisms, rhizobacteria-elicited induced systemic resistance (ISR) displays impressive resistance to a wide range of plant diseases

(Heil and Bostock 2002). As gram negative bacteria, *Pseudomonas* and *Serratia* groups are well known as typical ISR-inducing bacteria (van Loon et al. 1998). A *N*-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 and (2*R*, 3*R*)-butanediol and phenazine derivatives from *Pseudomonas chlororaphis* O6 have been reported as representative microbial metabolites exhibiting systemic resistance triggered by gram negative rhizosphere bacteria (Han et al. 2006; Ongena et al. 2005; Spencer et al. 2003).

Bacillus genus, a representative gram positive bacterium, has been intensively investigated on protection of plants against plant pathogens (Bais et al. 2004, Kloepper et al. 2004, Ongena et al. 2007;). Recently, *Bacillus* sp. BS107 (BS107) has been reported to exhibit ISR activity against a plant pathogen (Yang et al. 2009). However, bacterial determinant(s) from BS107 involved in ISR remain unknown. In the present study, we report for the first time 2-aminobenzoic acid as a microbial ISR-related compound, active against the soft-rot disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* SCC1 in tobacco. Among aminobenzoic acids tested in this study, 2- and 4-aminobenzoic acids showed ISR activity against soft-rot pathogen, but 3-aminobenzoic acid did not show the activity at the same treatment level.

Materials and methods

Chemicals

2-, 3-, and 4-aminobenzoic acids, salicylic acid, silica gel (Kieselgel, 70–230 mesh, 60 Å), and Sephadex LH-20 were purchased from Aldrich (Milwaukee, WI). Solvents used in this study were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA). Analytical grade chemicals were used throughout the study, unless otherwise stated.

Isolation of ISR determinant(s)

For isolation of an ISR-related metabolite, BS107 was grown in Tryptic Soy Broth (TSB) medium on a shaking incubator at 27°C for 2 days. The cultures were centrifuged at 10,000×g for 20 min and then the cultural filtrates were extracted twice with two times the volume of ethyl acetate. The organic extracts were dried in an EYELA model N-1000 evaporator at 40°C and dissolved in a solvent mixture of 80% (*v*/*v*) chloroform and 20% (*v*/*v*) methanol. An open glass column (15 mm i.d.×300 mm in length) was slurry-packed with silica gel in a solvent mixture of 80% (*v*/*v*) chloroform and 20% (*v*/*v*) methanol and the extracts were loaded onto the column. The column was eluted with the solvent mixture in which the methanol content was increased by 5% (*v*/*v*) at each step. Each eluate was evaporated in the evaporator at 40°C and subjected to the ISR bioassays as described below. The fractions containing ISR-active compounds were then subjected to Sephadex LH-20 column chromatography. An open glass column (15 mm i.d.×300 mm in length) was packed with Sephadex LH-20 in methanol and washed with four times the volume of methanol. The ISR fraction was eluted with methanol and each fraction was dried as described above. The ISR-active fractions were then injected onto a prep-HPLC (Dionex P680 dual pump, USA) equipped with a Dionex PDA-100 photodiode array detector at 254 nm. The preparative HPLC column was a μ-Bondapak C18 stainless column (7.8 i.d.×300 mm in length). The solvent system was 22% (*v*/*v*) aqueous methanol, supplemented with 0.1% (*v*/*v*) trifluoroacetic acid, and the flow rate was 2.0 mLmin^{-1}. The HPLC fraction with ISR activity was used for determination of the ISR compound.

ISR bioassays

ISR bioassays against soft-rot disease caused by *P. carotovorum* subsp. *carotovotrum* SCC1 (SCC1) were performed as previously described (Park et al. 2008). Briefly, the seeds of wild type (*Nicotiana tabaccum* L. cv. Xanti-nc) and NahG transgenic tobacco were disinfected in 70% (v/v) aqueous ethanol for 1 min followed by soaking in 0.5% (v/v) sodium hypochlorite for 1 min. The seeds were then rinsed with distilled water several times. The sterilized seeds were placed onto MS (Murashige and Skoog basal medium, Duchefa, USA) agar, composed of vitamins 0.5% (*v*/*v*) phyto agar and 3.0% (*v*/*v*) sucrose, and germinated in a growth chamber at 25°C with a 14 h light/10 h dark cycle for 5 days. The tobacco seedlings were transferred onto sterilized microtitre plates. Three weeks after transplanting, the seedling roots were treated with water, as a negative control, 1 mM salicylic acid, as a positive control, the authentic aminobenzoic acid isomers, bacterial cell treatment of strain BS107 at 10^8 colony forming units per ml, and the ISR-active

compound isolated from BS107. Salicylic acid was used as a positive control for the ISR assays because it is known to be a typical compound causing disease resistance against plant pathogens. Five days after treatment with the above substances, the leaves were treated with 2.0 μl of SCC1 suspensions (1×10^8 CFU ml^{-1}). After 3 days, disease severity was assessed by scoring disease symptoms as previously described (Park et al. 2009).

Reverse transcriptase (RT)-PCR

Total RNA was isolated from leaf tissues challenged with chemicals or SCC1 as described previously (Kim et al. 2006). Total RNA was treated with 1 U of RNase-free DNase (Bioneer, Korea) containing 1 μg of DNase-treated total RNA, oligo primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA). PCR reactions were carried out according to the manufacturer. The candidate priming genes were analyzed using the following primers: 5 5'-AAT ATC CCA CTC TTG CCG-3' (*PR1a*-F) and 5'-CCT GGA GGA TCA TAG TTG-3' (*PR1a*-R). Additional genes and the primer sets used to detect them were as follows: *PR1b*, 5'-ATC TCA CTC TTC TCA TGC-3' and 5'-TAC CTG GAG GAT CAT AGT-3'; *PR1c*, 5'-CTT GTC TCT ACG CTT CTC-3' and 5'-AAC ACG AAC CGA GTT ACG-3'; *PR2*, 5'-ACCAT CAGA CAAGATGT-3' and 5'-TGGCTAAGAGTG GAAGGT-3'; *PR4*,5`-ATGGTTGGAACTTCCGGA-3' and 5'TCCTGATCTCTCTGCTAC-3'; *PR5*, 5'-AT GAGAAAGACCCACGTC-3' and 5'-ATGCCT TCTTTGCAGCAG-3'; *PR6*,5`-ATGCCACAATCT CAACCA-3` and 5`-ACCTAATGCAGCCCGAAT-3`; *SAR8.2*,5`-CCTTGCCTTTCTTTGGCT-3` and 5`-GA CATTTAGGACATTTGCTGC-3` As a control to ensure that equal amounts of RNA were analyzed in each experiment, *Actin* was analyzed using the primers 5'-TGGACTCTGGTGATGGTGTC-3' and 5'-CC TCCAATCCAAACACTGTA-3'. Candidate priming genes were amplified from 100 ng of cDNA by PCR using an annealing temperature of 55°C. Amplified PCR products were separated by 2% agarose gel electrophoresis.

Growth inhibition assays

To rule out the possibility on direct inhibition of SCC1 by 2-AB, the growth inhibition assays of SCC1 was performed on TSB agar plates containing by 2-AB at of 10 to 1,000 $\mu g ml^{-1}$. SCC1 was grown on TSB media to reach A600 1.5 and diluted two times in TSB media. A 100 μl-aliquot of SCC1 suspensions was spread onto the TSB plates. The growth of SCC1 was investigated on the basis of colony forming unit (CFU) values in 48 h. The aqueous methanol was used as a solvent control.

Instrumental analyses

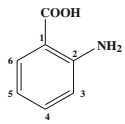
To estimate accurate mass of the isolated metabolite, a Bruker Daltonics Q-TOF mass spectrometer (TOF-MS) was used. The HPLC system was interfaced to the TOF-MS equipped with electrospray ionization (ESI) interface in negative ion mode. LC/MS accurate mass spectra was scanned ranged from 50 to 800. The column was a μ-Bondapak C18 stainless column (2.0 i.d.× 150 mm in length). The solvent system was the same as described above with a flow rate of 0.5 $ml min^{-1}$. A Shimazu model QP2010 GC/MS system equipped with a DB-5 capillary column (0.25 i.d.×30 m in length, 1.0 μm film thickness) was also used. The carrier gas was helium and the flow rate was 1.0 ml min^{-1}. Injection was performed in a split mode of 50:1. The initial column temperature was set for 2 min at 100°C, followed by ramping at a rate of 10°C min^{-1} to 280°C. The injector temperature was 280°C. Mass spectrometry was performed in the chemical ionization mode with *iso*-butane as a chemical gas and electron impact mode with ionizing voltage 70 eV. GC/MS chemical library databases (Willey7, NIST27 and NIST147) were used in order to characterize the ISR-active compound. A Varian model Unity INOVA 500 MHz NMR spectrometer was used for ^1H analysis of the isolated metabolite.

Results

Identification of an ISR determinant of BS107

Q-TOF MS analysis of the isolated metabolite indicate a $C_7H_7NO_2$ compound with a $(M-H)^+$ peak at 136.0390. ^1H NMR spectra characterized protons of the isolated metabolite as presented in Table 1. The NMR data demonstrated four different benzene protons at δ 7.86 (1H, dd), δ 7.31 (1H, td), δ 6.85 (1H, dd), and δ 6.73 (1H, td). The NMR data of the

Table 1 NMR data of 2-aminobenzoic acid isolated from BS107

Structure	Position	δ^1H (ppm)	Multiplicity	Coupling constant (Hz)	
				^3J	^4J
	3	7.86	1H, dd	7.2	1.2
	4	7.31	1H, td	7.2	1.2
	5	6.85	1H, dd	7.2	1.2
	6	6.73	1H, td	7.2	1.2

isolated metabolite were the same as the data of commercial authentic 2-aminobenzoic acid (2-AB). Protons of carboxylic acid and amine were not detected in both the isolated 2-AB and the authentic 2-AB, probably due to the hydrogen bonding interaction between the NMR solvent (CD_3OD) and the chemicals. HPLC analysis of cell-free culture of BS107 detected 2-AB at the same retention time as that of authentic 2-AB (Fig. 1a). GC/MS analysis of the isolated 2-AB showed an M^+ peak at 137 in EI mode (Fig. 1b). The base peak at 119 was due to the loss of water molecule from the parent ion peak at

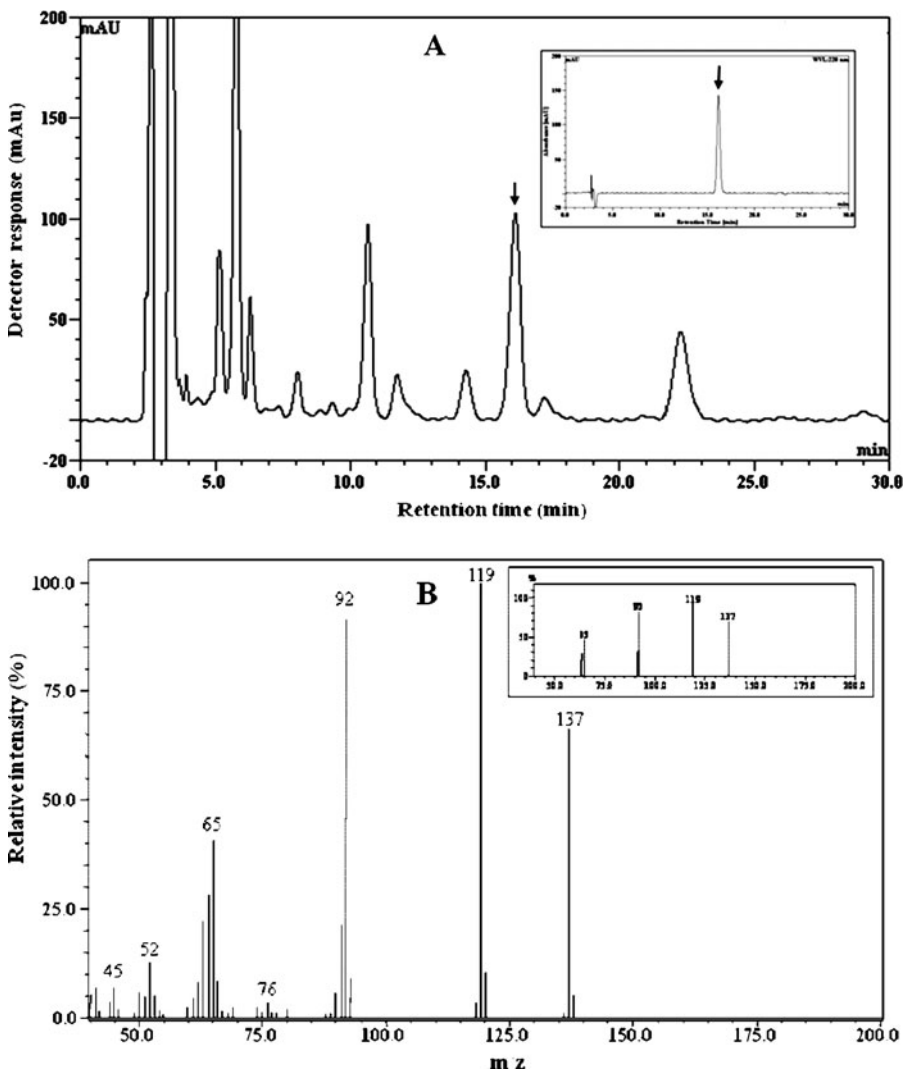


Fig. 1 HPLC chromatograms of cell-free culture of BS107 (**a**) and GC/MS spectra of 2-aminobenzoic acid isolated from BS107 in EI (**b**) mode. The insets represent respective data of authentic 2-aminobenzoic acid. The arrow symbols represent 2-AB detected

137, and the fragment ion peaks at 92 and 65 were resulted from subsequent loss of carbonyl and ethylene from the base peak at 119, which resembled those of the authentic 2-AB. The resulting mass spectrum analysis showed 98% similarity to 2-aminobenzoic acid based on the chemical library databases. Taken from the data of MS and NMR, the ISR compound isolated from BS107 was identified to be 2-AB. One may consider if 2-AB would be already present in the TSB medium and if the concentration of 2-AB produced by BS107 would not be realistic. For these, 2-AB was determined in the TSB extracts and time-course determination of 2-AB in cell-free cultures during the incubation was performed by TOF MS at a mode of extracted ion chromatogram. No detectable 2-AB was observed in the TSB extracts. In addition, TOF MS analysis of cell-free cultures detected the increased 2-AB with incubation time (Fig. 2). These suggest that 2-AB was not present in the TSB medium, but was produced by BS107.

ISR bioassay of 2-AB and its isomers

Treatment with cell-free cultures of BS107 resulted in approximately 15% disease incidence, while treatment with water as a negative control resulted in nearly 87% disease incidence (Fig. 3). When the plant seedling roots were treated with the authentic 2-AB at concentration levels of 2.3 and 23 mM, the disease incidence rates were about 37 and 11%, respectively. 2-AB at 23 mM showed the ISR activity at a level similar to 1.0 mM salicylic acid as a positive control. The ISR bioassays were also carried out with 3- and 4-ABs because they are aminobenzoic acid isomers. Table 2 shows disease incidence rates in tobacco seedlings inoculated with chemicals at different concentrations. The treatment with 2-AB or 4-AB significantly reduced the disease symptom development, while the tobacco seedlings treated with water showed about 70% disease incidence. Significant phytotoxicity was only observed in the tobacco seedlings treated with 3-AB at the same treatment levels as those of other isomers. The growth inhibition assays suggested no direct contact inhibition of SCC1 by 2-AB (data not shown).

PR genes expression

To obtain molecular evidences to elicitation of ISR by 2-AB treatment after application of pathogen or chemicals for testing direct influence on defense gene expression and after pathogen challenge for indirectly

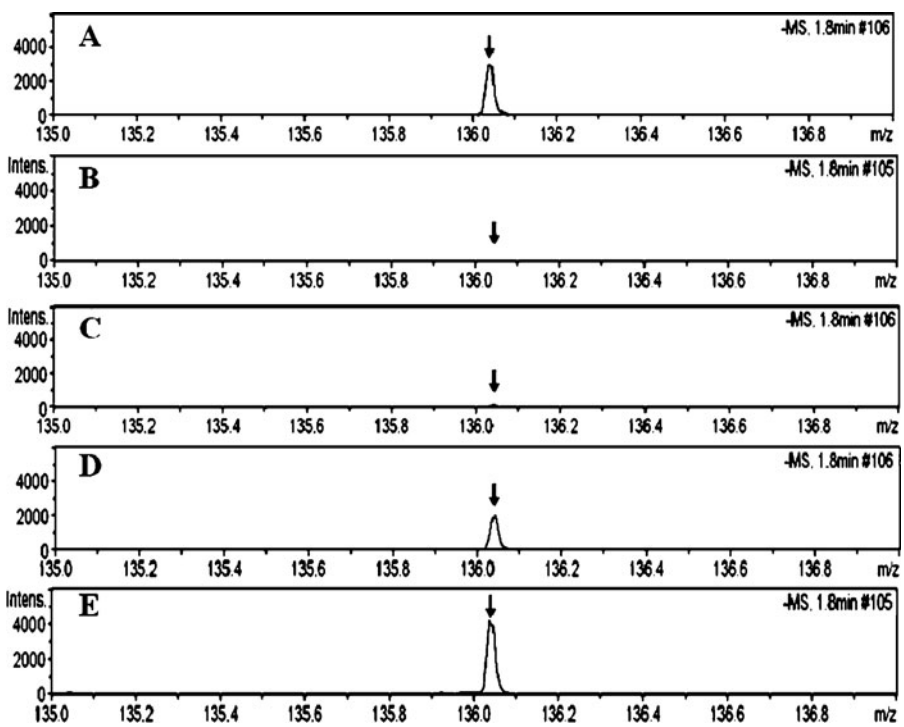


Fig. 2 TOF MS extracted ion chromatograms of 0.04 mM of authentic 2-AB (**a**), the TSB medium extracts (**b**) and cell-free cultures of BS107 incubated for 0 (**c**), 24 (**d**) and 36 h (**e**), respectively. The arrow symbols represent 2-AB detected or not. The cell-free cultures were diluted 20 times with the TSB after incubation and injected onto HPLC-TOF MS

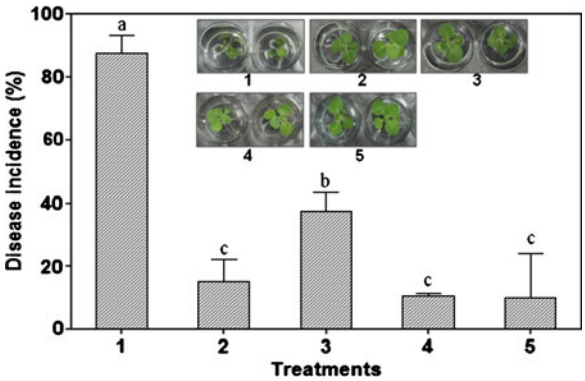


Fig. 3 Disease incidence values of tobacco seedlings treated with water (1) as a negative control, cell-free cultures of BS107 (2), 2.3 mM 2-AB (3), 23 mM 2-AB (4) and 1.0 mM salicylic acid (5) as a positive control. Disease incidence (%) was evaluated 3 days after *P. carotovorum* subsp. *carotovorum* SCC1 inoculation. Two independent experiments were performed with 12 seedlings per treatment. Differences in letters indicate statistically significant differences between the treated and the control samples according to Duncan's multiple test ($P<0.05$) with SPSS 17.0 software program

assessment of potentiating defence gene induction referred to as "priming" by the treatments, we employed RT-PCR assays of transcriptional expression of PR genes against SCC1. The *PR1a* and *PR2* genes, marker genes for salicylic acid-dependent signal pathway, were strongly expressed, as compared to control, at 1 and 3 days after root drench-applications of 2-AB and BTH (Fig. 4). 2-AB and BTH treatments increased slightly the expression of the *PR1a* and *PR2* genes at 3 h after challenging with SCC1, indicating the two genes were primed by 2-AB treatment. The expression of the *PR2* gene was up-regulated by strain BS107 at 5 dpi. The induction of the *PR4* gene was observed at 3 dpi by 2-AB and at 6 hpi by SCC1. The expression of *PR1c* was increased at 1, 3 and 5 dpi by 2-AB and BTH treatments, while the expression of *PR5* and *PR6* genes was not different from the control. Collectively, disease suppression by application of 2-AB can be caused by systemic induction of defence genes such as *PR1a*, *PR1c*, *PR2*, and *PR4* rather than direct inhibition of pathogenic bacteria cells.

ISR assessment in NahG transgenic tobacco

To validate salicylic acid-dependent signaling pathway, we used NahG transgenic plants carrying a bacterial *nahG* gene encoding salicylate hydroxylase that degrades SA to catechol, an inactive form (Dempsey et al., 1999). Salicylic acid treatment at 1.0 mM significantly reduced disease severity as 0.75 while lost the capacity when treated in NahG plants. 2-AB application at 1.0 and 18.0 mM into NahG plants showed significantly 2.5- and 2.8-fold higher symptom development, respectively, than that of wild type plants (Fig. 5). However, 2-AB elicited ISR compared to control when analyzed only in the NahG tobacco plants. Our results indicate that 2-AB-mediated ISR is depending on SA-dependent signalling.

Discussion

In this study, we describe the isolation and identification of an ISR determinant secreted by BS107 that previously reported the induction of systemic resistance in plant. 2-AB produced by BS107 displayed effective ISR-activity against soft-rot disease caused by SCC1 on the tobacco leaves. The ISR elicitor properties of 2-AB were demonstrated on the basis of 1) treatments of tobacco roots with the isolated 2-AB and authentic 2-AB exhibited the protective effects against the plant pathogen SCC1, 2) 2-AB did not

Table 2 Disease incidence values of tobacco seedlings treated with aminobenzoic acids

Treatments	Disease incidence values (%)[a]
Water	70.8±4.2
2-Aminobenzoic acid (1.0 mM)	16.7±4.8
2-Aminobenzoic acid (18.0 mM)	17.0±7.3
3-Aminobenzoic acid (1.0 mM)	–[b]
4-Aminobenzoic acid (1.0 mM)	33.0±5.0
4-Aminobenzoic acid (18.0 mM)	16.6±4.8
Salicylic acid (1.0 mM)	8.7±2.9

[a] Data are means±SD of three determinations

[b] Significant phytoxicity was observed

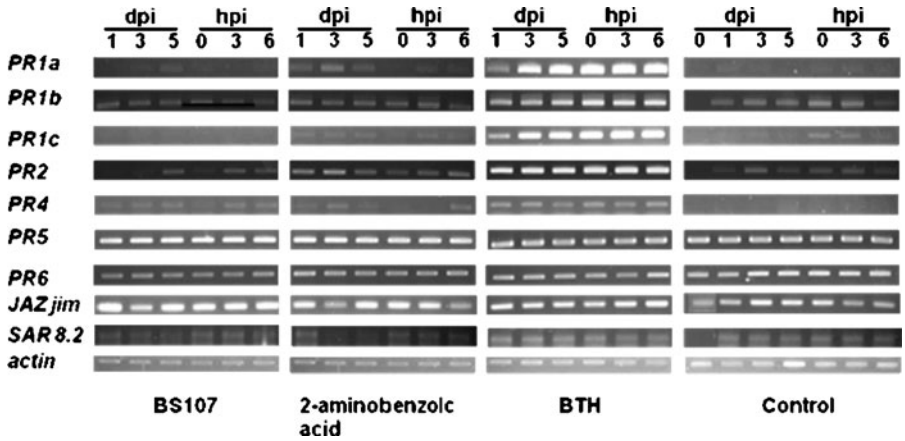


Fig. 4 Elicitation of defense-related gene expression by 2-AB in tobacco. The expression of PR genes (*PR1a*, *PR1b, PR1c, PR2, PR4*, *PR5*, *PR6*, *JAZ-jim*, and *SAR8.2*) analyzed by RT-PCR was examined at 0, 1, 3, and 5 days post-inoculation (dpi) of 2-aminobenzoic acid on tobacco root and 0, 3, and 5 h post-inoculation (hpi) of *P. carotovorum* subsp. *carotovorum* SCC1 on the leaves. Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. As a positive control, plants were treated with 0.5 mM BTH. The experiment repeated two times with similar results

inhibit the growth of SCC1 at the concentrations that exhibited ISR activity, 3) treatment of tobacco roots revealed up-regulation of the induced resistance marker genes such as *PR1a*, *PR1c*, *PR2* and *PR4*. Among 2-AB isomers tested at 1.0 mM, 4-AB exhibited similar ISR activity as did 2-AB, while 3-AB showed the lack of ISR at the same treatment level. The data suggest that the *meta*-substituted isomer seems to have ISR activity lower than the *ortho*- or *para*-substituted isomers. Further studies to investigate the mechanisms resulted in the different ISR responses between the isomers would be of interest.

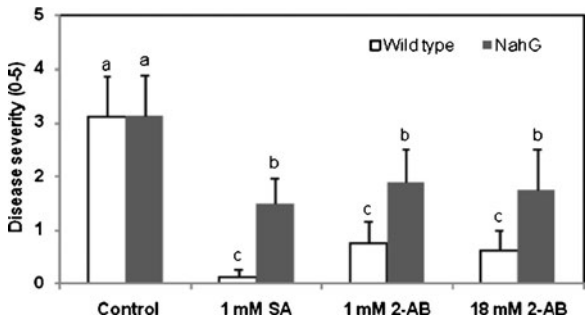


Fig. 5 Induced systemic resistance of wild type and NahG transgenic tobacco against *P. carotovorum* subsp. *carotovorum* SCC1 by 2-AB. Numbers represent mean of 10 replications per treatment, one seedling per replication. 2-AB at 1.0 and 18.0 mM were inoculated in the potting media contains 3-week-old seedlings of wild type (cv. Xanthi-nc) and NahG transgenic tobacco. Salicylic acid solution (1.0 mM) was used as a positive control. Disease severity (0–5) was measured 48 h after pathogen challenge. Different letters indicate significant differences using Fisher's protected LSD test at P=0.05. This experiment was conducted two times

2-AB is biosynthesized from chorismic acid, which is a precursor for salicylic acid. Salicylic acid is known to play a role in mediating plant defences (Audenaert et al. 2002). It seems, therefore, that 2-AB acid would be responsible for induced systemic resistance via a salicylic acid-dependent mechanism against soft-rot disease in tobacco. We confirmed the results by finding that 2-AB protected systemically NahG tobacco against *P. carotovorum* subsp. *carotovorum*, indicating that 2-AB-elicitged ISR requires SA signalling (Fig. 5). In another viewpoint, 2-AB is known to be a growth inhibitor of roots in *Arabidopsis thaliana* (Hoang et al. 2007), in which 2-AB is converted to indole acetic acid. This phenomenon gives rise to increased ethylene content within plant, which would express ethylene-dependent genes (Ryu et al. 2004) to induce systemic resistance to plant pathogens (Ryu et al. 2003; Spencer et al. 2003). In growth-inhibition bioassays, 2-AB showed no direct antimicrobial activity against SCC1, suggesting that they seem to be converted to any key compound related to ISR activity or to trigger plant systemic defence mechanisms in tobacco (Yamakawa et al. 1998). Furthermore, RT-PCR assessment revealed the up-regulation of induced resistance marker genes such as *PR1a*, *PR2*, and *PR4*, indicating

that 2-AB elicits ISR via salicylic acid and ethylene-dependent signalling pathways (Yang et al. 2009). The result indicates that we cannot exclude the possibility of involvement of other bacterial determinants from strain BS107 on ISR in this system. Intriguingly, as with our previous result in pepper plants, 2-AB treatment primed the *PR4* gene for ISR after pathogen challenge in tobacco (Fig. 4). Taken from, our results indicate that 2-AB elicited ISR by modulation of plant systemic-defense signalling.

Based on our findings, colonization of PGPR such as BS107 on the plant root system can play a role in promoting plant defences, as it secretes bacterial determinants including 2-AB for protecting plants against challenge by diverse plant pathogens. 2- AB and 4-AB may serve as an approach to investigate the biochemical changes in plant responsiveness to pathogen challenge.

Acknowledgement This work is supported by a grant (20100301-061-185-001-03-00) of the Biogreen 21 program from the Rural Development Administration, Republic of Korea.

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