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Identification of an ISR-Related Metabolite Produced by *Pseudomonas chlororaphis* O6 against the Wildfire Pathogen *Pseudomonas syringae* pv. *tabaci* in Tobacco

Park, Myung Ryeol[1], Young Cheol Kim[1], Ju Yeon Park[1], Song Hee Han[1], Kil Yong Kim[1], Sun Woo Lee[2], and In Seon Kim[1]*

[1]*Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Korea*
[2]*Department of Chemistry, College of Natural Science, Chonnam National University, Gwangju 500-757, Korea*

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***Pseudomonas chlororaphis* O6 exhibits induced systemic resistance (ISR) against *P. syringae* pv. *tabaci* in tobacco. To identify one of the ISR metabolites, O6 cultures were extracted with organic solvents, and the organic extracts were subjected to column chromatography followed by spectroscopy analyses. The ISR bioassay-guided fractionation was carried out for isolation of the metabolite. High-resolution mass spectrometric analysis of the metabolite found $C_9H_9O_3N$ with an exact mass of 179.0582. LC/MS analysis in positive mode showed an $(M+H)^+$ peak at *m/z* 180. Nuclear magnetic resonance (^1H, ^{13}C) analyses identified all protons and carbons of the metabolite. Based on the spectroscopy data, the metabolite was identified as 4-(aminocarbonyl) phenylacetate (4-ACPA). 4-ACPA applied at 68.0 mM exhibited ISR activity at a level similar to 1.0 mM salicylic acid. This is the first report to identify an ISR metabolite produced by *P. chlororaphis* O6 against the wildfire pathogen *P. syringae* pv. *tabaci* in tobacco.**

Keywords: Induced resistance, ISR, *Pseudomonas chlororaphis* O6, *Pseudomonas syringae* pv. *tabaci*

The control of plant pathogens by plant-associated microorganisms may involve direct growth inhibition of pathogens and induction of defense mechanisms in the plant. Plant defenses generally are controlled through two pathways, the salicylic acid (SA) pathway and the jasmonic acid (JA) pathway [1–3, 9]. Microbial metabolites active as systemic inducers include phenazines from the root-colonizing microbe *Pseudomonas chlororaphis* O6, which induces resistance to *Erwinia carotovora* subsp. *carotovora* [7], and an *N*-alkylated benzylamine derivative from *P. putida* BTP1 [11]. Certain synthetic chemicals, such as isonicotinic acid and benzothiadiazole, are used commercially as substitutes for SA in inducing disease resistance [12]. Considering that the induced defense system is one of the methods that allow plants to protect themselves from plant pathogens, finding new systemic resistance inducers is of a great interest in agriculture, emphasizing the use of environmentally safe chemicals.

Korea has a strong environmentally friendly organic farming (ENOF) program that has helped to decrease the use of synthetic pesticides by as much as 40% in agriculture since 1995. Microbiological approaches for crop protection against plant pathogens are a strategy of the ENOF program in Korea. The induction of plant immune systems by ISR compounds may play a role in decreasing the use of synthetic pesticides in the control of plant pathogens.

Pseudomonas chlororaphis O6 is one of the pseudomonads exhibiting ISR activity against plant pathogens, including *P. syringae* pv. *tabaci* in tobacco [13]. The production of 2*R*, 3*R*-butanediol by strain O6 appears to be a major factor in resistance against the soft-rot pathogen *E. carotovora* subsp. *carotovora* SCC1. However, microbial metabolites of strain O6 exhibiting ISR activity against the wildfire pathogen *P. syringae* pv. *tabaci* remain unknown. Here, we aimed to identify a metabolite from O6 cultures that induces resistance to leaf infection by *P. syringae* pv. *tabaci* in tobacco.

The ISR against the wildfire pathogen *P. syringae* pv. *tabaci* was assayed as described earlier [4, 5, 13]. Tobacco seeds were surface-disinfected in 70% ethanol for 1 min, followed by soaking in 1% sodium hypochlorite for 30 sec. The seeds were rinsed several times with distilled water. The surface-disinfected seeds were placed on mineral salt (MS) agar, consisting of 0.5% (v/v) agar and 3%

*Corresponding author
Phone: 82-62-530-2131; Fax: 82-62-530-2139;
E-mail: mindzero@chonnam.ac.kr

sucrose, in microtiter plates and germinated in growth chambers at 25°C with a photoperiod of 14 h light:10 h darkness for 2 weeks. Roots were treated with water or a suspension of *P. chlororaphis* O6, as described previously [4, 5]. After 5 days, the leaves were spot-inoculated with 2 μl of *P. syringae* pv. *tabaci* prepared at 1×10^8 colony forming units/ml on each leaf, and disease rates were assessed 3 days after challenge with the plant pathogen, as described previously [13].

For isolation of ISR-related metabolites, *Pseudomonas chlororaphis* O6 was grown in 8 l of Luria-Bertani (LB) medium supplemented with streptomycin and ampicillin at 50 mg/l, respectively, on a shaking incubator at 27°C until the cultures were orange, indicating the production of the secondary metabolites, phenazines [13]. The cultures were then centrifuged at 10,000 ×*g* for 15 min to pellet the cells, and the supernatant was extracted with equal volumes of ethyl acetate and *n*-butanol. These organic phases were discarded, and the aqueous phase was adjusted to pH 2 by addition of 1 N HCl solution and extracted again with *n*-butanol. The *n*-butanol fraction was evaporated to dryness in an evaporator at 60°C, and the residue was dissolved in 30% (v/v) aqueous methanol for ODS column chromatography. An open glass column (33 mm×750 mm in length) was slurry-packed with ODS in methanol and subsequently washed with four times the column bed volumes of methanol, distilled water, and 30% (v/v) aqueous methanol. The *n*-butanol fraction obtained above was then loaded into the column. The column was eluted with two times the column bed volumes of a solvent mixture consisting of methanol and water, where the concentration of methanol was increased with each wash. Each fraction was evaporated in an evaporator at 50°C and subjected to ISR assays as described in the previous section. The ISR-related fractions were collected and freeze-dried, and the dried extracts were dissolved in distilled water and purified through a series of solvent washes on a Supelco C_{18} SPE cartridge column (Bellefonte, PA, U.S.A.). The solvent mixture contained acetonitrile and water, and the concentration of acetonitrile was increased with each wash. The SPE washing fractions were concentrated under a gentle stream of nitrogen gas and then subjected to the ISR assays as described above. For further purification, the ISR-related fractions obtained from SPE cartridges were injected into a reversed-phase semi-prep HPLC (Dionex P680 dual pump) equipped with a Dionex PDA-100 photodiode array detector at 190, 270, and 380 nm. The preparative HPLC column was a μ-Bondapack C_{18} stainless column (7.8 mm× 300 mm, 10 μ film thickness). The HPLC mobile phase consisted of 10% (v/v) aqueous acetonitrile adjusted to pH 2 by trifluoroacetic acid, and the column was eluted at 4 ml/min. Main peaks detected were collected separately and subjected to ISR assays as described above. The leading edge and tail of each peak were excluded to reduce possible contamination. The purified metabolite was analyzed by a Jeol model JMS 700 High Resolution Mass spectrometer (HRMS) coupled with a QQHQC-type mass analyzer to determine the molecular formula and exact mass of the metabolite isolated. A Hewlett Packard model 1100 HPLC coupled with a VG Quattro LC triple quadrupole tandem mass spectrometer was also used to investigate mass spectra of the isolated metabolite. A Varian model Unity INFINITYplus 200 mHz nuclear magnetic resonance (NMR) spectrometer and a Varian model Unity INOVA 500 mHz NMR were used for the ^{13}C and ^{1}H analyses of the isolated metabolite, respectively.

Pseudomonas chlororaphis O6 exhibits induced systemic resistance (ISR) against two foliar bacterial pathogens, *P. syringae* pv. *tabaci* and *Erwinia carotovora* subsp. *carotovora* [5, 8, 10, 13]. 2*R*, 3*R*-butanediol produced by strain O6 is a microbial metabolite exhibiting ISR to the soft-rot leaf pathogen *E. carotovora* subsp. *carotovora* SCC1, but not against the wildfire leaf pathogen *P. syringae* pv. *tabaci* [4]. *Pseudomonas chlororaphis* O6 is also a bacterium capable of producing phenazines with characteristic orange pigmentation [7]. A mutation in the global regulator *gac*S gene of strain O6 eliminates phenazine production and does not prevent the induction of ISR activity against *P. syringae* pv. *tabaci* in tobacco [13]. However, the induction of systemic resistance to *E. carotovora* subsp. *carotovora* SCC1 is impaired by the *gac*S mutant [13]. These results suggest that inducers other than 2*R*, 3*R*-butanediol and phenazines may be involved in the ISR of strain O6 against *P. syringae* pv. *tabaci* in tobacco. We, therefore, aimed to identify one of the microbial metabolites from O6 cultures that exhibits induced resistance to leaf infection by *P. syringae* pv. *tabaci* in tobacco.

The ISR bioassay-guided fractionation of O6 cultures yielded an ISR metabolite that was identified by spectroscopy analyses including HRMS, LC/MS, ^{13}C-NMR, and ^{1}H-NMR. HRMS analysis of the isolated metabolite revealed $C_9H_9O_3N$ with an exact mass of 179.0582. LC/MS analysis of the isolated metabolite in positive mode identified an $(M+H)^+$ peak at *m/z* 180 (Fig. 1). Table 1 shows the data from ^{1}H- and ^{13}C-NMR analyses of the isolated metabolite. ^{13}C-NMR analysis detected seven carbon signals that contained one methyl carbon at C 24.14, two carbonyl carbons at C

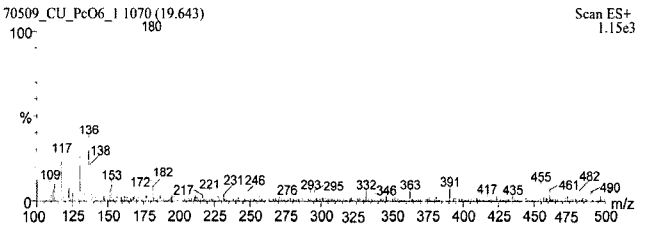

Fig. 1. Positive-ion LC/MS spectra of the ISR metabolite isolated from *Pseudomonas chlororaphis* O6 cultures.

Table 1. NMR spectra data of the ISR metabolite isolated from *Pseudomonas chlororaphis* O6 cultures.

Carbon position	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	Coupling constant (Hz)	Multiplicity
1	24.14	2.15(3H)	-	s
2	172.11	-	-	-
3	144.52	-	-	-
4	120.18	7.65(2H)	J=8.5	d
5	131.90	7.95(2H)	J=8.5	d
6	127.51	-	-	-
7	172.11	-	-	-
NH_2	-	Not detected	-	-

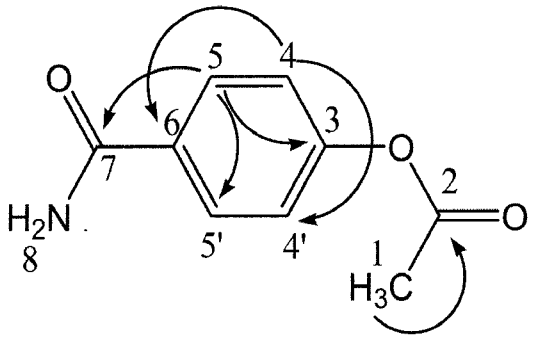

Fig. 2. Chemical structure and HMBC correlations of the ISR metabolite isolated from *Pseudomonas chlororaphis* O6 cultures.

172.11, two quaternary carbons at C 144.52 and 127.51, and two olefinic methine carbons at C 120.28 and 131.90. ^{1}H-NMR analysis detected three protons of the methyl group at H 2.15 (s), and four olefinic methine protons at H 7.65(d), and 7.95(d). Protons of carboxylic amide were not detected, probably because of a hydrogen bonding interaction between the NMR solvent (CD_3OD) and the isolated metabolite. The isolated metabolite was very soluble in water, *n*-butanol, and methanol, but not in nonpolar solvents such as acetone, and pure acetonitrile. Based on the data of mass spectrometry and NMR analyses, the isolated metabolite was identified as 4-(aminocarbonyl) phenylacetate (4-ACPA). The chemical structure and HMBC correlations of 4-ACPA are presented in Fig. 2.

To investigate whether 4-ACPA exhibits the ISR against *P. syringae* pv. *tabaci*, ISR activity assays were performed in tobacco leaves. Fig. 3 shows the disease symptoms of the leaves of tobacco seedlings after exposure to *P. syringae* pv. *tabaci*. Spreading areas of necrosis were observed on the leaves of control tobacco plants where inocula of *P. syringae* were administered (Fig. 3A). On the other hand, necrosis was reduced just to the site of infection in the plants treated with SA (Fig. 3B), which was also observed when the plants had roots colonized by *P. chlororaphis* O6 and 4-ACPA (Figs. 3C and 3D). Applications of 4-ACPA (68.0 mM) generated an ISR response similar to that for SA (1.0 mM). These results indicate that 4-ACPA is one of the microbial metabolites of O6, exhibiting induced systemic resistance to leaf infection by *P. syringae* pv. *tabaci*. The pathway by which the isolated metabolite is generated in the O6 strain remains to be elucidated.

Here, we report for the first time that 4-ACPA produced by strain O6 is a new ISR metabolite. We found that 4-ACPA was produced in both the wild-type O6 strain and its *gac*S mutant (data not shown). *Gac*S controls the synthesis of

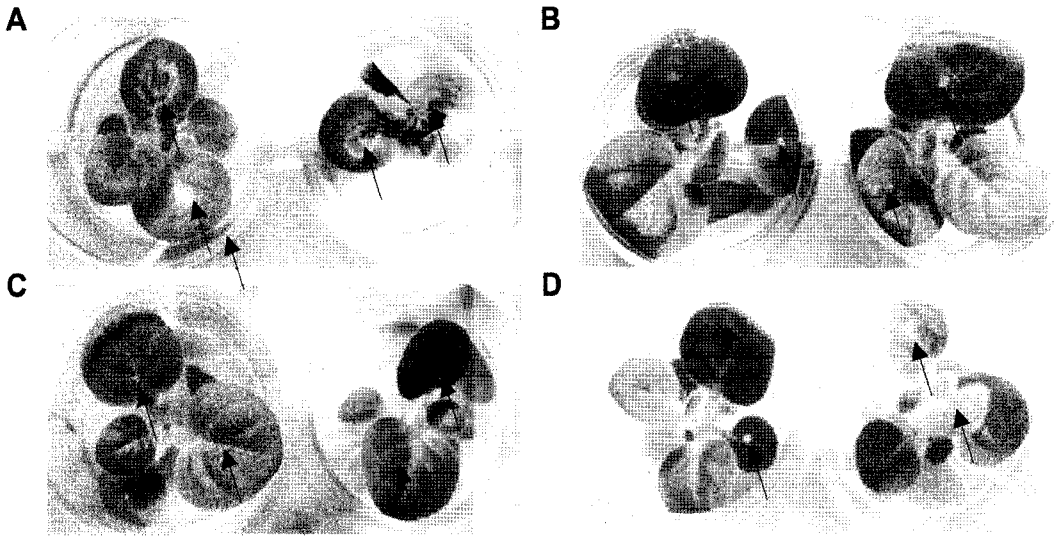

Fig. 3. Effects of root treatment with water as a control (**A**), salicylic acid (**B**), strain O6 (**C**), and the isolated ISR elicitor (**D**) on wildfire symptoms on tobacco leaves.

The leaves were challenged with *P. syringae* pv. *tabaci* 5 days after treatments with the substances described above. Arrows denote where drops of the pathogen were placed.

phenazine and 2*R*,3*R*-butanediol, which are also ISR inducers. The production of 2*R*,3*R*-butanediol has been correlated with ISR against the soft-rot pathogen in O6, but this induced pathway was not effective against *P. syringae* pv. *tabaci* [4]. From our findings, we conclude that *P. chlororaphis* O6 can produce several metabolites that are significant in inducing systemic resistance. 4-ACPA is one of the ISR metabolites produced by the O6 strain. Resolving the pathways for induced resistance will reveal where the overlaps and differences occur in remodeling of plant defenses by these metabolites.

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