GacS-Dependent Production of 2R, 3R-Butanediol by *Pseudomonas chlororaphis* O6 Is a Major Determinant for Eliciting Systemic Resistance Against *Erwinia carotovora* but not Against *Pseudomonas syringae* pv. *tabaci* in Tobacco

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Root colonization by a plant-beneficial rhizobacterium, *Pseudomonas chlororaphis* O6, induces disease resistance in tobacco against leaf pathogens *Erwinia carotovora* subsp. *carotovora* SCC1, causing soft-rot, and *Pseudomonas syringae* pv. *tabaci*, causing wildfire. In order to identify the bacterial determinants involved in induced systemic resistance against plant diseases, extracellular components produced by the bacterium were fractionated and purified. Factors in the culture filtrate inducing systemic resistance were retained in the aqueous fraction rather than being partitioned into ethyl acetate. Fractionation on high-performance liquid chromatography followed by nuclear magnetic resonance mass spectrometry analysis identified the active compound as 2R, 3R-butanediol. 2R, 3R butanediol induced systemic resistance in tobacco to *E. carotovora* subsp. *carotovora* SCC1, but not to *P. syringae* pv. *tabaci*. Treatment of tobacco with the volatile 2R, 3R-butanediol enhanced aerial growth, a phenomenon also seen in plants colonized by *P. chlororaphis* O6. The isomeric form of the butanediol was important because 2S, 3S-butandiol did not affect the plant. The global sensor kinase, GacS, of *P. chlororaphis* O6 was a key regulator for induced systemic resistance against *E. carotovora* through regulation of 2R, 3R-butanediol production. This is the first report of the production of these assumed fermentation products by a pseudomonad and the role of the sensor kinase GacS in production of 2R, 3R-butanediol.

Additional keywords: promotion of plant growth, volatile compounds.

Durable, broad-spectrum resistance against foliar or systemic bacterial diseases has been shown to be induced by the colonization of the rhizosphere by certain nonpathogenic bacterial

strains (Anderson et al. 2005; Ryu et al. 2004b; Spencer et al. 2003; Van Loon et al. 1998; Yang et al. 2002). Many of the bacterial traits exhibited by these nonpathogenic rhizobacteria have been associated with the induction of systemic resistance (Lugtenberg et al. 2001; Meziane et al. 2005; Van Loon et al. 1998). Compounds relevant to this phenomenon include the O-antigenic lipopolysaccharide from *Pseudomonas fluorescens* in the tomato plant (Duijff et al. 1997), the flagellins (Zipfel et al. 2004), the pyoverdines from fluorescent *Pseudomonas* spp., and salicylic acid (SA) produced under iron-limited conditions (Meziane et al. 2005). An N-alkylated benzylamine derivative from *P. putida* BTP1 has been recognized as an effective inducer of systemic resistance against *Botrytis cinerea* in bean (Ongena et al. 2005). Recently, two volatile compounds, acetoin and butanediol, produced by gram-positive *Bacillus* sp. strains, were shown to induce systemic resistance in tobacco against a soft-rot pathogen, *Erwinia carotovora* SCC1 and promote plant growth (Ryu et al. 2003, 2004a). These results indicate that a variety of bacterial components are involved in induction of systemic resistance, a phenomenon associated with protection of plant species against a range of potential pathogens.

The present article is focused on the traits of the root-colonizing *P. chlororaphis* O6 that are involved in the induction of systemic resistance. The colonization of cucumber roots by this bacterium confers protection against corynespora spot caused by *Corynespora cassicola* (Kim et al. 2004). Colonization of tobacco roots also induces resistance in the leaves against the soft-rot pathogen *E. carotovora*, as well as the wildfire-associated pathogen, *P. syringae* pv. *tabaci* (Spencer et al. 2003). However, although the global sensor kinase of *P. chlororaphis* O6, GacS, is required for the induction of resistance against *E. carotovora*, it is not required for the induction of protection against *P. syringae* pv. *tabaci* (Spencer et al. 2003). In subsequent studies, we have observed that colonization of tobacco roots by *P. chlororaphis* O6 stimulates aerial growth approximately threefold (Kang et al. 2006). In this study, we report that *P. chlororaphis* O6 produces 2R, 3R-butanediol, like the *Bacillus* isolates studied by Ryu et al. (2003, 2004a). We confirmed that 2R, 3R-butanediol stimulates aerial growth and the induction of resistance to the soft-

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rot pathogen in plants colonized by *P. chlororaphis* O6 but not against *P. syringae* pv. *tabaci*. Our study of the role of the *gacS* gene on the regulation of butanediol formation provides an explanation for the observation that the *gacS* gene is not required for the induction of resistance in tobacco against *P. syringae* pv. *tabaci* (Spencer et al. 2003).

RESULTS

Purification of an extracellular ISR determinant from *P. chlororaphis* O6.

Induced systemic resistance (ISR) against *E. carotovora* pv. *carotovora* SCC1 was observed in tobacco plants when the roots were colonized by *P. chlororaphis* O6. Whereas 83% of leaves of noncolonized tobacco showed symptoms of soft rot, only 13% of leaves in the colonized plants developed symptoms. We did not detect cells of *P. chlororaphis* O6 from the aerial portions of the plants when these were transferred to King's medium B (KB) agar plates. Similarly, in other studies with bean, barley, and *Arabidopsis* seedlings grown from seed inoculated with *P. chlororaphis* O6, we have not seen colonization of aerial tissues, only the root and seed surfaces.

Fractionation of the components in the culture filtrates of *P. chlororaphis* O6 showed that the ISR-active material was retained in the aqueous fraction and was not partitioned into ethyl acetate (Figs. 1 and 2). The ethyl acetate fraction contained the yellow-colored phenazines that had antifungal activity (Spencer et al. 2003). The ISR-active materials eluted

from silica gel chromatography with 70% ethyl acetate and 30% methanol. Subsequent purification by LH-20 Sephadex chromatography yielded ISR-active material at an elution volume per total volume (V_e/V_t) of approximately 0.7 to 0.9. High-performance liquid chromatography (HPLC) analysis of this material revealed four peaks; only peak 4 possessed ISR activity (peak 1, t_R 7.78 min; peak 2, t_R 8.44 min; peak 3, t_R 11.49 min; peak 4, t_R 13.11 min) (Figs. 1 and 3).

Analysis of the peak 4 fraction by a gas-chromatograph electron-impact mass spectrometer (GC/EI/MS) revealed a molecular ion (M^+) with mass-to-charge ratio (m/z ; relative intensity) of 90 (1.6%) (Fig. 3). The mass of other ions formed in the fragmentation were 75 [$(M-CH_3)^+$, 3.2%], 57 [$(M-(CH_3, H_2O))^+$, 9.2%], and 45 [$(M-CH(OH)CH_3)^+$, 100%]. The resultant mass spectrum was identical to that of butanediol, which has been cataloged as NIST147 (NIST147, LIB entry 1120; CAS: 6982-25-8). When MS analysis was conducted using authentic 2R, 3R-butanediol, the spectrum obtained (90 [0.4%], 75 [2.4%], 57 [9.6%], 45 [100%]) was identical to that of the ISR compound from *P. chlororaphis* O6 (Fig. 3).

Butanediol has three stereoisomers: 2R, 3R-butanediol, 2S, 3S-butanediol, and 2R, 3S-butanediol. The HPLC peak 3 (t_R 11.01 min) and peak 4 (t_R 13.11 min) were consistent with those of the standards, 2S, 3S-butanediol (t_R 11.42 min) and 2R, 3R-butanediol (t_R 13.16 min), respectively. Thus, *P. chlororaphis* O6 produced both 2R, 3R and 2S, 3S forms of butanediol. In planta activity was observed only with the 2R, 3R-butanediol.

Culture broth of *Pseudomonas chlororaphis* O6 (2L)

Fig. 1. Fractionation protocol and yield information for extracellular products from *Pseudomonas chlororaphis* O6 that induce systemic protection against the soft-rot pathogen in tobacco. *P. chlororaphis* O6 cells were grown in Luria Bertani broth (2 liters) for 48 h to stationary-phase.

Role of 2R, 3R-butanediol in ISR and the promotion of plant growth.

We confirmed that authentic 2R, 3R-butanediol, at a dose range of 1 mg to 100 µg/plant, induced systemic resistance to the soft-rot pathogen, *E. carotovora*, in the tobacco plant (Table 1). The maximum level of protection was achieved by a dosage of 100 µg/root. Treatment with dosages of less than 10 µg/root was ineffective at induction of systemic resistance. 2S, 3S-butanediol applied at 100 µg/root did not induce systemic resistance against the soft-rot disease.

Protection against the soft-rot pathogen was observed in plants with roots colonized by *P. chlororaphis* O6 and for plants with roots treated with 2R, 3R-butanediol. The extent of protection was similar to that conferred by treatment with SA, benzothiodiazole (BTH), and *P. fluorescens* 89B-61 (Fig. 4). However, when plants treated with authentic 2R, 3R-butanediol were challenged with the wildfire pathogen, *P. syringae* pv. *tabaci*, no protection was observed (Fig. 5A and B). Resistance to the wildfire pathogen was observed in the plants treated with SA and BTH or plants with roots colonized by *P. chlororaphis* O6 and *P. fluorescens* 89B-61.

Tobacco seedlings exposed to the volatiles produced during the growth of *P. chlororaphis* O6, or to authentic 2R, 3R-butanediol, displayed increased leaf surface area (Fig. 6A). No effects on the growth of plants raised in I plates, which contain partitions to separate plants and treatments, were detected by

applications of SA, BTH, or an inoculum of *P. fluorescens* 89B61 (Fig. 6B).

GacS is a key regulator of ISR activity against *E. carotovora* because it is required for butanediol production.

ISR in tobacco against soft rot required a functional *gacS* gene in the *P. chlororaphis* O6 cells colonizing the roots (Fig. 7A). ISR was detected when roots were colonized by the wild-type or the *gacS*-complemented mutant (Fig. 7A). No protection was observed when roots were colonized by the *gacS* mutant. When I plates were used to separate plants that were inoculated with *P. chlororaphis* O6 from those that were not, protection was conferred in the noninoculated plants only when the other set of plants was inoculated with the wild-type or the complemented *gacS* strains (Fig. 7A and B). No cross-protection was afforded to the noninoculated plants when the I plates contained plants inoculated with the *gacS* mutant. These studies with the I plates illustrated that it was a volatile material that induced resistance and suggested that a functional GacS was required for butanediol formation by the pseudomonad.

The regulation of butanediol formation by GacS was confirmed by purification of culture filtrates from the *gacS* mutant through the same procedures that were used with the wild-type strain (Fig. 1). The peaks eluted from the HPLC column corre-

Fig. 2. Resistance of the leaves of tobacco plants to *Erwinia carotovora* SCC1 in plants with and without root colonization by *Pseudomonas chlororaphis* O6, or after the treatment with the ethyl acetate-extracted (EtoAc phase) or water-soluble components (Aqueous phase) from *P. chlororaphis* O6 culture filtrates. Root treatments and disease assessment were conducted with seedlings grown in well plates. Photographs were taken 2 days after inoculation with *E. carotovora* SCC1. Arrows illustrate the collapse of leaves in nonprotected plants because of the development of soft rot.

Fig. 3. High-performance liquid chromatography (HPLC) chromatograms of **A**, 10 µM 2S, 3S-butanediol as a standard, **B**, the extracellular materials from *Pseudomonas chlororaphis* O6 after fractionation by Sephadex column chromatography, and **C**, 100 µM 2R, 3R-butanediol as a standard. **D**, The mass spectrum analysis obtained by electrospray ionization of the induced systemic resistance-active peak 4 from the HPLC fractionation of the wild-type products. Au = absorbance units.

sponding to the butanediols from the wild-type strain were absent in the extracts obtained from the *gacS* mutant (Fig. 8). However, other peaks of unknown materials were detected in the extracts from the *gacS* mutant (Fig. 8).

DISCUSSION

Systemic resistance against the soft-rot pathogen induced by root colonization with *P. chlororaphis* O6 in tobacco was correlated with the production of 2R, 3R-butanediol. This isomer, as well as the 2S, 3S-butanediol isomer, was detected in the culture filtrates of this bacterium after growth in liquid Luria Bertani (LB) medium. The butanediols also were likely to be produced on defined medium, based on our detection of ISR from the volatiles emitted during *P. chlororaphis* O6 growth on defined medium in I plates. Only 2R, 3R-butanediol was active,

Table 1. Effect of dose of authentic 2R, 3R-butanediol on induced systemic resistance in tobacco against *Erwinia carotovora* subsp. *carotovora* SCC1^y

Dose of 2R, 3R-butanediol (µg/plant)	Disease incidence (%) ^z
0	58.0 ± 8.0 d
1,000	13.3 ± 4.6 ab
100	10.7 ± 4.6
10	19.0 ± 5.2 abc
1	19.0 ± 5.2 abc
0.1	24.7 ± 8.5 bc
0.01	22.0 ± 5.2 abc
0.001	24.7 ± 8.5 bc
0.0001	27.7 ± 4.6 c
0.00001	52.7 ± 4.6 d

^y Seedlings for this study were grown in well plates and treated with 10 µl of 2R,3R-butanediol at defined concentrations. Leaves were challenged with the soft-rot pathogen and disease levels scored after 2 days. Control plants were treated with water. Three independent experiments were conducted with 21 plants/treatment.

^z Differences in letters indicate a significant difference between treatments.

Fig. 4. Induced systemic resistance against soft-rot pathogen *Erwinia carotovora* SCC1 in tobacco seedlings. Seedlings grown in well plates had roots inoculated with *Pseudomonas chlororaphis* O6 (O6) or *P. fluorescens* 89B61 (89B61), or were treated with 10 µl of authentic 100 µM 2R, 3R-butanediol (Butanediol), 1 mM salicylic acid (SA), 100 µM benzothiodiazole (BTH), or water as a control (control). After 1 week, leaves were challenged with the pathogen and disease was assessed 2 days later. Three independent experiments were conducted with 21 plants/treatment. Differences in letters indicate significant differences between the treated and control samples, based on Duncan's multiple range test at $P = 0.05$.

with detectable responses being registered with root treatments as low as 100 pg/root. The 2S, 3S-isomer was inactive at 100 µg/root, the dose shown to be the peak concentration for the 2R, 3R-isomer.

The production of 2R, 3R-butanediol by *P. chlororaphis* O6 could account for the increase in aerial growth of the tobacco plants upon root colonization by this bacterium (Kang et al. 2006). Thus, the pseudomonad *P. chlororaphis* O6 has properties reminiscent of *Bacillus* spp. in promoting plant growth and inducing systemic protection against challenge with the soft-rot pathogen (Ryu et al. 2003, 2004a). In both the bacillus and *P. chlororaphis* O6, these traits are correlated with 2R, 3R-butanediol generation.

To the best of our knowledge, this study is the first report of butanediol formation by a pseudomonad. The mechanism by which the 2R, 3R butanediol exerts its effects in the plant remains to be clearly elucidated, but an intact ethylene signaling pathway is required for both *P. chlororaphis* O6 and the chemical to elicit the responses (Ryu et al. 2004a; Spencer et al. 2003). Although 2R, 3R-butanediol conferred protection against the

Fig. 5. Induced systemic resistance against the wildfire pathogen *Pseudomonas syringae* pv. *tabaci* in tobacco seedlings. **A**, Seedlings were grown in pot culture and the roots were inoculated with 10 ml of *P. chlororaphis* O6 (O6) or treated with 10 ml of 100 µM authentic 2R, 3R-butanediol or 2S, 3S-butanediol, or with water as control. The pathogen *P. syringae* pv. *tabaci* (10^8 CFU/ml) was infiltrated into the leaves with a sterile syringe. Images of the leaves were taken at 5 days after challenge with *P. syringae* pv. *tabaci*. Arrows show the development of necrosis typical of the onset of disease caused by this pathogen. Results are typical of 10 leaves on five plants in studies that were replicated twice. **B**, Seedlings were grown in well plates on Murashige and Skoog salt medium and roots were inoculated with *P. chlororaphis* O6 (O6) or *P. fluorescens* 89B61 (89B61) or treated with 100 µM authentic 2R, 3R-butanediol (Butanediol), 1 mM salicylic acid (SA), 100 µM benzothiodiazole (BTH), or water (control). Suspension of the pathogen *P. syringae* pv. *tabaci* (10^8 CFU/ml) was dropped onto the tobacco leaves. The extent of disease symptoms were recorded 5 days after treatment. Data are from three independent experiments, each with 21 plants/treatment. Differences in letters indicate significant differences between the treated and control samples, based on Duncan's multiple range test at $P = 0.05$.

soft-rot pathogen, no protection was induced against the wild-fire pathogen. This finding helps explain our observation (Spencer et al. 2003) that a functional *gacS* gene was essential for the protection induced by *P. chlororaphis* O6 against the soft-rot pathogen, but not against the wildfire-causing pathogen. In this article, we demonstrate that an intact *gacS* gene was required for butanediol synthesis. Purification of the aqueous extracts from the *gacS* mutant of *P. chlororaphis* O6 showed no peaks in the final HPLC analysis that correlated with either of the butanediols produced by the wild-type strain. Peaks with different retention times were detected at the final stage of purification through the C-18 column for the extracts from the *gacS* mutant; however, the nature of the materials is not known.

The control over butanediol synthesis exerted by the sensor kinase GacS extends the list of properties that are controlled by this regulator. The Gac system in pseudomonads governs the production of the diffusible signaling molecules, the acyl homoserine lactones, which in turn regulate an array of factors involved in bacterial survival (Haas and Keel 2003; Spencer et al. 2003). This potential role of quorum sensing in the regulation of butanediol synthesis is interesting with regard to the control of acetoin biosynthesis by a complex quorum sensing system in *Vibrio cholera* (Kovacicova et al. 2005). Here, acetoin biosynthesis is negatively regulated by AphA, which plays a crucial role in the regulation of the expression of virulence-associated genes. The production of

the uncharged butanediol and acetoin from glucose is believed to help limit intracellular acidification in *V. cholera* (Kovacicova et al. 2005).

The pathway by which these compounds are generated in pseudomonads remains to be elucidated in detail. The biosynthesis of butanediol in lactobacilli involves mixed-acid fermentations of pyruvate (Maestri and Joset 2000). Similarly, in *Bacillus* spp., the presence of a pathway involving anaerobic fermentation from pyruvate was indicated by the results of mutational analysis revealing the functions of acetolactate synthase and acetolactate decarboxylase (Grundy et al. 1993; Ryu et al. 2003). Genes encoding acetolactate synthase are present in all of the genomes for *Pseudomonas* spp. *putida* and *fluorescens*, plant-pathogenic *syringae*, and broad-range pathogenic *aeruginosa* in the JGI-Integrated Microbial Genomes data base. Genes identified as encoding putative 2,3-butanediol dehydrogenase enzymes, which would convert acetoin to the butanediols, are identified in a subset of these genomes: *P. aeruginosa*, *P. fluorescens* Pf-5, and *P. putida* KT2440 and F-1. However, genes predicted to encode enzymes involved in acetoin degradation by other pathways also are reported within the pseudomonad genome database. We currently are examining the *P. chlororaphis* O6 genome for genes involved in acetolactate, acetoin, and butanediol metabolism.

Fig. 6. Plant growth promotion of tobacco seedlings by volatiles from *Pseudomonas chlororaphis* O6 (O6), *P. fluorescens* 89B61 (89B61), authentic 100 µM 2R, 3R-butanediol (Butanediol), 1 mM salicylic acid (SA), 100 µM benzothiodiazole (BTH), or water (control). Bacterial strains or chemicals were present on the opposite side of the I plates dishes to the tobacco plant. **A**, Photographs were taken at 10 days after bacterial treatment. **B**, Total leaf surface area of each leaf measured at 10 days after bacterial treatments. Three independent experiments with 21 plants/treatment were performed. Differences in letters indicate a significant difference between treated and control samples based on Duncan's multiple range test at $P = 0.05$.

Fig. 7. Effect of a *gacS* mutation in *Pseudomonas chlororaphis* O6 on induced systemic resistance against *Erwinia carotovora* in the tobacco plant. Tobacco plants were inoculated with wild-type *P. chlororaphis* O6 (O6), the *gacS* mutant (GacS-), or the complemented *gacS* mutant (Com GacS-), or treated with water as a control. Treatments were **A**, applied directly to the roots or **B**, used to generate volatiles and tested on I plates. At 7 days after these treatments, the leaves were challenged with *E. carotovora*, and disease incidence was recorded 2 days later. Three independent experiments were conducted with 21 plants/treatment. Differences in letters indicate significant differences between the treated and control samples, based on Duncan's multiple range test at $P = 0.05$.

Of particular importance to our work is the volatility of these compounds, meaning that their effects may be distant from the bacterial cell of origin. Our studies have uncovered another aspect of signaling from pseudomonads within the rhizosphere, involving the process by which bacterial volatiles are sensed and generate beneficial responses in the plant.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacteria were stored at -70°C in 15% glycerol. The construction of the *gacS* mutant and a *gacS*-complemented strain of *P. chlororaphis* O6 was as described by Spencer and associates (2003). The *gacS* mutant was complemented in trans with a 6.3-kb *Bam*HI-*Kpn*I *gacS*-containing fragment from the wild type housed in the broad-host-range plasmid pCPP46(Tc^R).

These strains were grown at 28°C with shaking at 200 rpm in either KB (King et al. 1954) or LB broth (Sambrook and Russel 2001) for routine culturing. The pseudomonads used for seed inoculation were grown to an optical density at 600 nm ($\text{OD}_{600\text{nm}}$) = 2.0 in LB broth, centrifuged, and then resuspended in sterile water to 1×10^8 CFU/ml. When appropriate, bacteria were cultured in media supplemented with antibiotics: ampicillin (Ap) at 100 $\mu\text{g/ml}$ and streptomycin at 50 $\mu\text{g/ml}$ for growth of *P. chlororaphis* O6 wild type, and kanamycin (Km) (the *gacS* mutant) at 50 $\mu\text{g/ml}$ and tetracycline (Tc) at 20 $\mu\text{g/ml}$ for the complemented-*gacS* mutant. A strain *P. fluorescens* 89B61 that induces systemic resistance was provided by J. Kloepper (Auburn University, AL, U.S.A.) and was used as a positive control in this study. The *Arabidopsis* pathogen *E. carotovora* subsp. *carotovora* SCC1 was acquired from E. Tapio Palva (University of Helsinki, Finland). The wildfire-causing pathogen *P. syringae* pv. *tabaci* was provided by J. W. Kloepper (Auburn University).

Isolation and fractionation of ISR-active materials from *P. chlororaphis* O6.

P. chlororaphis O6 and *gacS* mutant cells were grown in LB broth (2 liters) for 48 h to stationary phase. The cultures were centrifuged for 15 min at 5,000 rpm at 4°C . The supernatant was adjusted with 1 N HCl to pH 3.0 and extracted three times with an equal volume of ethyl acetate. The organic and aqueous phases were concentrated in vacuo and dissolved in water in order to assay their potential for the induction of systemic resistance. The ISR-active aqueous fractions were fractionated on a silica gel column (1.7×58 cm, Kieselgel 60, 70 to 230 mesh, 60 g; Merk, Darmstadt, Germany), via stepwise elution with increasing concentrations of methanol in ethyl acetate. The ISR-active fraction was purified by Sephadex LH-20 column chromatography (2.2×80 cm, 25 to 100 mesh; Pharmacia Fine Chemicals, Uppsala, Sweden) with methanol at a flow rate of 1.5 ml/min. The active eluates were subjected to HPLC using a Waters Delta prep 4000 Preparative Chromatography System (Waters Inc., Milford, MA, U.S.A.). The analytical column was a μ -Bondapak C₁₈ stainless column (7.8×300 mm, 10 μm in diameter; Waters Inc.). Fractions were eluted at a flow rate of 1.0 ml/min, with a mobile phase of methanol/water (70:30, vol/vol). Materials in the elute were detected at 220 nm with a Waters 2487 Dual λ absorbance Detector (Waters Inc.). Authentic 2R, 3R-butanediol and 2S, 3S-butanediol, (Sigma-Aldrich, Milwaukee, WI, U.S.A.), were applied to the column as standards. These stages of the purification are outlined in Figure 1.

Identification of the ISR-active compound via GC/EI/MS.

The ISR-active compounds were analyzed using a GC/EI/MS (GCMS-QP2010, 70 eV; Shimadzu, Kyoto, Japan)

equipped with a GC (GC2010; Shimadzu) under the following conditions: column, VB-1 (60 m by 0.25 mm; Gig Harbor, WA, U.S.A.) coated with 100% demethylpolysiloxane; carrier gas, helium; and column flow rate of 0.94 ml/min. The injector and ion source were maintained at 200°C . The column temperature was programmed to 30°C for 3 min, elevated at 5°C/min to 180°C and by 40°C/min to 200°C , and then maintained for 2 min at 200°C . The sample was injected with an ionizing voltage of 70 eV. The mass range was scanned at 0.5 atomic mass unit (amu)/s from 40 to 350 amu. The Wiley7, NIST27, and NIST147 library databases were searched in the attempt to identify the ISR-active compound. Authentic 2R, 3R-butanediol also was subjected to mass spectrophotometric analysis to confirm the fragmentation pattern.

Plant materials.

Tobacco cv. Xanthi seed were stored at 5°C and were surface-sterilized via 2 min of washing in sterile water, followed by 10 min of soaking in 75% ethanol and 10 min of immersion in 3% (vol/vol) sodium hypochlorite. The seeds were rinsed four times in an excess of sterile distilled water, and then held in sterile tubes for 2 days of vernalization at 4°C in darkness. Seed were placed singly into wells series of 12-well plates (SPL Inc., Seoul, Korea), each containing one-half-strength Murashige and Skoog salt medium (GIBCO-BRL, Rockville, MD, U.S.A.) amended with 0.5% Phyta gel and 3% sucrose, adjusted to pH 5.7.

For the assessment of ISR activity against the wildfire pathogen, the sterilized seed were planted into sterile soil-less medium (peat moss/vermiculite/perlite, 7:3:3, vol/vol/vol) using 500 cm³ of this matrix in a 10.5-by-10.5-by-9-cm pot, with 15 to 30 seeds per pot. The seedlings were grown with a cycle of 16 h of light and 8 h of dark under 40-W fluorescent lights (80 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). The temperature was maintained at $26 \pm 1^{\circ}\text{C}$ with a relative humidity of 60%. Seedlings were watered with 20 ml of sterile water per pot at 2-day intervals.

Fig. 8. High-performance liquid chromatography (HPLC) chromatograms of extracellular products from **A**, *Pseudomonas chlororaphis* O6 wild type and **B**, the *gacS* mutant. Fractions were prepared from the culture filtrates. Au = absorbance units.

Assessment of ISR against soft-rot and wildfire diseases.

Three weeks after seeding in microtiter plates, the roots were treated with either 10 μ l of bacterial cultures (1×10^8 CFU/ml), sterile water as negative control, or the extracted compounds at different purification stages. Additionally, the roots were treated with 10 μ l of 1 mM SA or 100 μ M BTH because of their abilities to induce systemic resistance in plants.

The pot-grown seedlings were treated after 4 weeks of growth. The roots of each seedling were treated with 10 ml of the suspension of *P. chlororaphis* O6 cells or aqueous solutions of 100 μ M 2R, 3R or 2S, 3S butanediol or water as a control.

One week after the administration of the bacterial or chemical treatments, the tobacco plants were subjected to challenge with *E. carotovora* subsp. *carotovora* SCCI or *P. syringae* pv. *tabaci*. Most inoculations involved pipetting of 2 μ l of the bacterial inoculum onto each leaf. For the pot-grown plants challenged with *P. syringae* pv. *tabaci*, the inoculum was infiltrated into the leaf tissue. The phytopathogenic bacteria were grown for 48 h in LB broth and harvested with centrifugation, then washed in sterile water and resuspended in sterile distilled water at $OD_{600nm} = 1.0$ (1×10^8 CFU/ml). Leaves were treated with sterile distilled water as negative controls. Two days after pathogen challenge, soft-rot disease was rated by counting the number of leaves showing disease symptoms out of the 100 leaves treated. Symptoms were assessed 5 days after challenge with *P. syringae* pv. *tabaci*.

Evaluation of volatiles on the promotion of plant growth and ISR.

In order to test the role of volatiles in the promotion of plant growth and the induction of systemic resistance, sterilized tobacco seed were planted on one side of petri dishes, termed I plates, containing a center partition (SPL Inc.). Both sides of the I plates contained one-half-strength Murashige and Skoog salt supplemented with 1.5% (wt/vol) sucrose. After 2 weeks of tobacco growth, 10 μ l of bacterial suspensions, chemicals, or sterile distilled water were applied to the other side of each plate. Plates were sealed with Parafilm and incubated. One week after bacterial and chemical inoculations, total leaf surface area was measured using an automatic leaf area meter (Model AAM-9; Hayashi Denco Inc., Tokyo).

One week after bacterial or chemical treatments, tobacco plants were challenged with *E. carotovora* subsp. *carotovora* SCCI or *P. syringae* pv. *tabaci* by pipetting as described above. The disease incidences were rated.

Statistical analysis.

Data were analyzed by analysis of variance using SPSS 12.0K for Windows (SPSS Institute, Seoul, Korea). The significance of the treatments for plant protection was determined in accordance with the magnitude of the *F* value ($P = 0.05$). When a significant *F* test was obtained for treatments, the separation of means was accomplished by Fisher's protected least significant difference.

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