Induced Systemic Resistance in *Arabidopsis thaliana* Against *Pseudomonas syringae* pv. *tomato* by 2,4-Diacetylphloroglucinol-Producing *Pseudomonas fluorescens*

David M. Weller, Dmitri V. Mavrodi, Johan A. van Pelt,
Corné M. J. Pieterse, Leendert C. van Loon, and Peter A. H. M. Bakker

First author: United States Department of Agriculture–Agricultural Research Service, Root Disease and Biological Control Research Unit, Pullman, WA 99164-6430; second author: Department of Plant Pathology, Washington State University, Pullman 99164-6430; and third, fourth, fifth, and sixth authors: Plant–Microbe Interactions, Institute of Environmental Biology, Utrecht University, Padualaan 8, 3508 CH Utrecht, The Netherlands.
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ABSTRACT

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Pseudomonas fluorescens strains that produce the polyketide antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are among the most effective rhizobacteria that suppress root and crown rots, wilts, and damping-off diseases of a variety of crops, and they play a key role in the natural suppressiveness of some soils to certain soilborne pathogens. Root colonization by 2,4-DAPG-producing *P. fluorescens* strains Pf-5 (genotype A), Q2-87 (genotype B), Q8r1-96 (genotype D), and HT5-1 (genotype N) produced induced systemic resistance (ISR) in *Arabidopsis thaliana* accession Col-0 against bacterial speck caused by *P. syringae* pv. *tomato.* The ISR-eliciting activity of the four bacterial genotypes was similar, and all genotypes were equivalent in activity to the well-characterized strain *P. fluorescens* WCS417r. The 2,4-DAPG biosynthetic locus consists of the genes *phlHGF* and *phlACBDE. phlD* or *phlBC* mutants of Q2-87 (2,4-DAPG minus) were significantly reduced in ISR activity, and genetic complementation of the mutants restored ISR activity back to wild-type levels. A *phlF* regulatory mutant (overproducer of 2,4-DAPG) had ISR activity equivalent to the wild-type Q2-87. Introduction of DAPG into soil at concentrations of 10 to 250 μM 4 days before challenge inoculation induced resistance equivalent to or better than the bacteria. Strain Q2-87 induced resistance on transgenic NahG plants but not on *npr1-1*, *jar1*, and *etr1 Arabidopsis* mutants. These results indicate that the antibiotic 2,4-DAPG is a major determinant of ISR in 2,4-DAPG-producing *P. fluorescens*, that the genotype of the strain does not affect its ISR activity, and that the activity induced by these bacteria operates through the ethylene- and jasmonic acid-dependent signal transduction pathway.

Isolates of *Pseudomonas fluorescens* that produce the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) can be isolated from the rhizosphere environment of many plant species (24,38,39,64,65) and even marine environments (22). Within this group of bacteria, 22 distinct genotypes (A-T, PfY, and PfZ) have been described so far by either repetitive sequence-based polymerase chain reaction (rep-PCR) using the BOXA1R primer (BOX-PCR); restriction fragment length polymorphisms (RFLP) of *phlD*, a key gene in the 2,4-DAPG biosynthetic locus (*phlHGF* and *phlACBDE*); or phylogenetic analyses based on *phlD* sequences (9,30,31,37–39); however, several more genotypes occur (B. B. Landa and D. M. Weller, *unpublished data*).

2,4-DAPG-producing *P. fluorescens* isolates have been the subject of intense investigation because of their biocontrol activity against a wide range of plant pathogens (8,11,15,17,23,50,54,64) when applied as seed and soil treatments, and because they are responsible for the suppressiveness of certain soils against take-all disease of wheat (12,49,64), caused by *Gaeumannomyces graminis* var. *tritici*, and black root rot of tobacco (51,65), caused by *Thielaviopsis basicola.* 2,4-DAPG-producing pseudomonads also are enriched in the rhizospheres of wheat (65), pea (30), and flax (31) during monoculture to densities exceeding the threshold density required for pathogen suppression (10^5 CFU g^{-1} of root) (49), suggesting that these bacteria have a widespread role in plant protection (64,65).

2,4-DAPG has broad antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties (4,22,27,28,54). The key role that this antibiotic plays in disease suppression has been demonstrated both by studies using genetic mutational analysis with *P. fluorescens* strains CHA0, F113, Q8r1-96, Q2-87, and SSB17 (12,18,23,54,63) and by direct isolation of the antibiotic from rhizospheres colonized by these bacteria (7,23,47). 2,4-DAPG may either inhibit soilborne pathogens directly (7,8,11,12, 22,27,49) or induce systemic resistance in the host plant (21,56).

Rhizobacteria-mediated induced systemic resistance (ISR) is the ability of certain root-colonizing, nonpathogenic plant growth-promoting rhizobacteria to enhance (prime) the defensive capacity of the host against subsequent pathogen infection of foliage and roots that are spatially separated from the roots colonized by the inducing rhizobacteria (59). ISR is phenotypically similar to pathogen-induced systemic acquired resistance (SAR); both phenomena provide systemic resistance, reduce disease symptoms, inhibit pathogen growth in plant tissues, and operate in a variety of plant species (59). ISR acts through salicylic acid (SA)-dependent (10) or SA-independent mechanisms (44) and, in the latter, the signal transduction pathway is ethylene (ET) and jasmonic acid (JA) dependent.

Corresponding author: D. M. Weller; E-mail address: wellerd@wsu.edu

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Pseudomonas rhizobacteria activate ISR through different mechanisms that can be both complementary and additive and vary depending upon the pathosystem used as the assay (59). Bacterial determinants of ISR vary among pseudomonads (3,62) and include cell envelope components such as lipopolysaccharides (33,40,61) and flagella (40), antibiotics (1,21), lipopeptides (41,58), and iron-regulated metabolites such as siderophores (1,13,32,34,40) and SA (6,10,34). However, in some cases, SA may not function individually but, rather, is required as part of an SA-containing siderophore (3,52).

Iavicoli et al. (21) reported that root inoculation of *Arabidopsis thaliana* accession Columbia with *P. fluorescens* CHA0 protected leaves from *Hyaloperonospora arabidopsidis* and that production of 2,4-DAPG was required for ISR activity. Siddiqui and Shaukat (56) reported that 2,4-DAPG produced by CHA0 also induced systemic resistance in tomato against the root-knot nematode, *Meloidogyne javanica*. Strain CHA0 is a well-described 2,4-DAPG producer belonging to ARDRA group 1 and BOX-PCR genotype A (24,39,64) and produces a broad spectrum of bioactive metabolites in addition to 2,4-DAPG, including the antibiotics pyrrolnitrin and pyoluteorin (64). Strain CHA0 is very similar to the well-described strain Pf-5, whose genome has been sequenced (42).

The purpose of this study was to determine the ability of other genotypes of 2,4-DAPG producers to induce resistance in *A. thaliana* against bacterial speck caused by *P. syringae* pv. *tomato* and to identify the role that 2,4-DAPG plays in ISR activity. We demonstrated that *P. fluorescens* strains Pf-5, Q2-87, Q8r1-96, and HT5-1, representing four genotypes (A, B, D, and N, respectively), induced resistance to a similar level in *Arabidopsis*, and that these strains were equivalent in disease suppression to *P. fluorescens* WCS417r, a well-know ISR inducer. We also showed a key role for the antibiotic in ISR by these bacteria through a genetic mutational analysis of strain Q2-87 and direct application of 2,4-DAPG to the soil. A preliminary account of this work was previously published (66).

MATERIALS AND METHODS

Bacterial cultures and inoculum. Bacterial strains and plasmids used in this study are described in Table 1. 2,4-DAPG-producing *P. fluorescens* strains Pf-5, Q2-87, Q8r1-96, and HT5-1 were selected as inducers of resistance because they were isolated from three geographically different regions of the United States (Texas, Pf-5; Washington, Q2-87 and Q8r1-96; and Minnesota, HT5-1) and they represent two ARDRA groups (Pf-5, group 1; Q2-87, Q8r1-96, and HT5-1, group 2) and four genotypes (A, B, D, and N, respectively). *P. fluorescens* WCS417r (non-2,4-DAPG producer) was used as an ISR-positive control because it induces resistance in carnation, radish, and *A. thaliana* (45,60). The virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 (67), causal agent of bacterial speck, was the challenge pathogen. An avirulent derivative of DC3000 (*avrRpt2*), carrying plasmid pLAFR3 containing the avirulence gene *avrRpt2* (67), was used to induce SAR. *Escherichia coli* strain JM109 was used for all cloning experiments. Mutants and genetically complemented mutants of Q2-87 were prepared as described below, except for the 2,4-DAPG-minus mutant Q2-87::Tn*5*-1, which was previously constructed by Bangera and Thomashow (4). All strains except *E. coli* were rifampicin resistant, stored at –80°C, and cultured on King's medium B (KB) (25) or Luria-Bertani (LB) (2) agar or broth at 28°C or 37°C. Antibiotic supplements were used at the following concentrations: ampicillin, 40 or 100 μg ml^{-1}; rifampicin, 75 μg ml^{-1}; tetracycline, 12.5 or 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; chloramphenicol (13 μg ml^{-1}); and cycloheximide (100 μg ml^{-1}).

Bacterial inoculum for ISR assays was prepared essentially as previously described (45). Briefly, for soil inoculations, inducer

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[y]	Source or reference
Strains		
Pseudomonas fluorescens Pf-5	2,4-DAPG$^+$ Rif^r Genotype A[z]	20
P. fluorescens Q8r1-96	2,4-DAPG$^+$ Rif^r Genotype D[z]	49
P. fluorescens HT5-1	2,4-DAPG$^+$ Rif^r Genotype N[z]	39
P. fluorescens Q2-87	2,4-DAPG$^+$ Rif^r Genotype B[z]	63
P. fluorescens Q2-87::Tn*5*-1	2,4-DAPG$^-$ Rif^r Kan^r *phlD*::Tn*5*	4
P. fluorescens Q2-87BC	2,4-DAPG$^-$ Rif^r *ΔphlCB*::Tet^r	This study
P. fluorescens Q2-87D	2,4-DAPG$^-$ Rif^r *phlD*::Tet^r	This study
P. fluorescens Q2-87BCZ	2,4-DAPG$^-$ Rif^r *ΔphlCB*::*lacZ*	This study
P. fluorescens Q2-87DZ	2,4-DAPG$^-$ Rif^r *phlD*::*lacZ*	This study
P. fluorescens Q2-87F	2,4-DAPG$^+$ Rif^r *phlF*::Tet^r	This study
P. fluorescens WCS417r	2,4-DAPG$^-$ Rif^r	60
P. syringae pv. *tomato* DC3000	Rif^r	67
P. sryingae pv. *tomato* DC3000 *avrRpt2*	Rif^r pLAFR3 containing *avrRpt2*	67
Escherichia coli JM109	F' *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15/*recA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) mcrA*	Promega Corp.
E. coli S17-1(λ-pir)	*thi pro hsdR hsdM recA rpsL* RP4-2 (Tet^r::Mu) (Kan^r::Tn*7*) λ-pir	57
Plasmids		
pBluescript II KS	ColE1 f1(+) *bla*	Stratagene
pALTER-*Ex*1	ColE1 Tet^r SP6, *tac*, and T7 promoters	Promega Corp.
pNOT19	ColE1 *bla* accessory plasmid	53
pMOB3	Kan^r *cat oriT sacBR*	53
pSKS104	Source of promoterless *lacZYA* genes, ColE1 *bla*	55
pMON5122	RK2 Tet^r, pRK415 containing 7.08 kb *Bam*HI-*Xba*I fragment with *phlACBDE* genes	4
pPHL5122	pVS1 Kan^r, pVSP41 containing 7.08 kb *Bam*HI-*Xba*I fragment with *phlACBDE* genes	4
pMON5123	RK2 Tet^r, pRK415 containing 4.67 kb *Bam*HI fragment with *phlACBD* genes	4
pNOT-ΔNcoI-Tc^r-MOB	pNOT19 containing *phlCB*::Tet^r fusion ligated with MOB cassette	This study
pNOT-SalI-Tc^r-MOB	pNOT19 containing *phlD*::Tet^r fusion ligated with MOB cassette	This study
pNOT-SacI-Tc^r-MOB	pNOT19 containing *phlF*::Tet^r fusion ligated with MOB cassette	This study
pNOT-ΔNcoI-*lacZ*-MOB	pNOT19 containing *phlCB*::*lacZ* fusion ligated with MOB cassette	This study
pNOT-SalI-*lacZ*-MOB	pNOT19 containing *phlD*::*lacZ* fusion ligated with MOB cassette	This study

[y] 2,4-DAPG$^+$ or 2,4-DAPG$^-$ indicates that the strain does (+) or does not produce (–) 2,4-diacetylphloroglucinol; *bla,* β-lactamase; Kan^r, neomycin phosphotransferase; *tet* or Tet^r, tetracycline resistance; *cat*, chloramphenicol transferase; Rif^r, rifampin resistance.

[z] Genotypes were defined earlier by BOX polymerase chain reaction fingerprinting (39).

bacteria were grown for 24 to 36 h on KB at 28°C; cells from the plates were harvested into sterile 10 mM $MgSO_4$, washed, and suspended in 10 mM $MgSO_4$; and the concentration was appropriately adjusted based on optical density at 660 nm. *P. syringae* pv. *tomato* and avirulent *P. syringae* pv. *tomato* were grown for 20 to 24 h in KB broth, harvested by centrifugation, and suspended in 10 mM $MgSO_4$ and the concentration was appropriately adjusted.

DNA manipulations. Plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and *E. coli* transformation were carried out according to standard protocols (2). Electroporation of *Pseudomonas* spp. was performed according to Enderle and Farwell (16) with a Bio-Rad Gene Pulser system (Bio-Rad Laboratories, Hercules, CA). PCR amplifications were carried out with *Taq* DNA polymerase (Promega Corp., Madison, WI) according to the manufacturer's recommendations. DNA sequencing was performed with an ABI Prism BigDye Terminator v.3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Oligonucleotide primers were designed with Oligo 6.0 software (Molecular Biology Insights, Inc., Cascade, CO) and sequence data were analyzed with the OMIGA 2.0 software (Accelrys, San Diego, CA).

Construction of 2,4-DAPG biosynthetic and regulatory mutants. *P. fluorescens* Q2-87 2,4-DAPG-deficient mutants (Q2-87D, Q2-87BC, Q2-87DZ, and Q2-87BCZ), and regulatory mutant Q2-87F were generated by using a gene-replacement system described by Schweizer (53). To generate *P. fluorescens* Q2-87D and *P. fluorescens* Q2-87BC, a 4.67-kb *Bam*HI fragment of pMON5123 containing *phlACBD* (4) was subcloned into pBluescript II KS (Stratagene, La Jolla, CA). To generate the *phlCB* knockout, the plasmid was digested with *Nco*I and polished with a Klenow fragment of DNA polymerase I. The 517-bp *Nco*I fragment containing portions of the *phlC* and *phlB* genes was then replaced with a 2.5-kb *Pvu*II fragment bearing a tetracycline resistance gene from pALTER-*Ex*1 (Promega Corp.) (Fig. 1). Similarly, to generate the *phlD* knockout, the tetracycline resistance determinant from pALTER-*Ex*1 was introduced into the unique *Sal*I site within the *phlD* gene. The interrupted inserts were subcloned into pNOT19, and the plasmid was then digested with *Not*I and ligated with a 5.3-kb pMOB3 *sacB* cassette. To construct *P. fluorescens* Q2-87F, a 2.6-kb *Eco*RV fragment of pMON5123 containing *phlF* and *phlA* was subcloned into pNOT19, and the tetracycline resistance determinant from pALTER-*Ex*1 was introduced into the unique *Sac*I site within the *phlF* gene. The resulting plasmids (pNOT-ΔNcoI-Tc^r-MOB, pNOT-SalI-Tc^r-MOB, and pNOT-SacI-Tc^r-MOB) were mobilized into *P. fluorescens* Q2-87 through mating with *E. coli* S-17 (λ-pir). Transconjugants were selected on LB agar supplemented with rifampicin at 75 μg/ml and tetracycline at 25 μg/ml, and selection for double crossovers was carried out on LB agar supplemented with 5% sucrose. Following selection, all isolates were screened for the absence of plasmid-borne markers by Southern hybridization and PCR with specific primers (36).

P. fluorescens Q2-87BCZ and *P. fluorescens* Q2-87DZ strains were constructed essentially as described above, except that a 5.3-kb *Sal*I fragment from pSKS104 containing promoterless *lacZYA* genes was used to interrupt the *phlCB* and *phlD* genes (Fig. 1). The resulting plasmids, pNOT-ΔNcoI-lacZ-MOB and pNOT-SalI-lacZ-MOB, were mobilized into *P. fluorescens* Q2-87 through mating with *E. coli* S-17 (λ-pir). The integrity of the fusions was confirmed by DNA sequencing, PCR, and Southern hybridization. Overnight incubation of *P. fluorescens* Q2-87DZ and *P. fluorescens* Q2-87BCZ on KMB agar supplemented with X_{gal} and DAPG resulted in formation of blue colonies (data not shown).

Complementation of *P. fluorescens* Q2-87DZ and *P. fluorescens* Q2-87BCZ. For complementation studies, broad-host-range recombinant plasmids pMON5122 and pPHL5122 (Table 1) that bear *phlACBD* genes were introduced into strains Q2-87DZ and Q2-87BCZ by electroporation. Following the initial round of selection on LB agar supplemented with tetracycline or kanamycin, several randomly picked individual colonies were used to start cultures in KB broth. After 48 h of growth at 28°C, all cultures were analyzed for 2,4-DAPG and MAPG production by using high-performance liquid chromatography (HPLC) as described previously (4,7).

Cultivation of plants and inoculation. *A. thaliana* wild-type accession Col-0, transgenic NahG (impaired in SA-dependent defense response), and mutants *npr1-1* (impaired in both SA- and JA/ET-dependent defense responses), *etr-1* (ET insensitive), and *jar1* (JA insensitive) (14) were grown as previously described (26,52), with some modifications. These mutants were used to help determine the plant hormone signaling involved in ISR. Seed were germinated in plastic trays (14 by 29 cm) containing 2 kg of sterile quartz sand, supplemented with 200 ml of half-strength Hoagland nutrient solution (HNS) (19) containing 10 μM Fe-ethylene-diamine di-o-hydroxyphenylacetic acid (FeEDDHA) (Syngenta, Basel, Switzerland). Seed were sprinkled on the top of the sand and misted with water. The trays were placed in larger covered trays (28.5 by 43.5 cm) to maintain the relative humidity at 100% and incubated in a climate chamber or greenhouse with an 8-h day and 16-h night cycle at 24 and 20°C, respectively, and at 60 to 70% relative humidity. Seedlings (10 to 14 days old) were individually transplanted into 60-ml plastic pots (one seedling/pot), each containing ≈100 g of a potting soil/sand mixture, which was autoclaved twice for 30 min and then supplemented with HNS + FeEDDHA (50 ml/kg of mix) and with either 10 mM $MgSO_4$ with bacteria (10^9 CFU ml^{-1}) or an equal amount of 10 mM $MgSO_4$ as a control (50 ml/kg of mix). Pots were placed in the large trays, and the seedlings were grown for an additional 3 weeks in the climate chamber. HNS + FeEDDHA was applied to the pots once a week, and tap water was given when needed.

ISR bacterial treatments consisted of wild-type strains, mutants, and genetically complemented mutants of strain Q2-87 thoroughly distributed throughout the potting mix (introduced bacteria at 5×10^7 CFU g^{-1} of mix). Pure 2,4-DAPG also was tested for ISR activity at concentrations of 10, 100, and 250 μM.

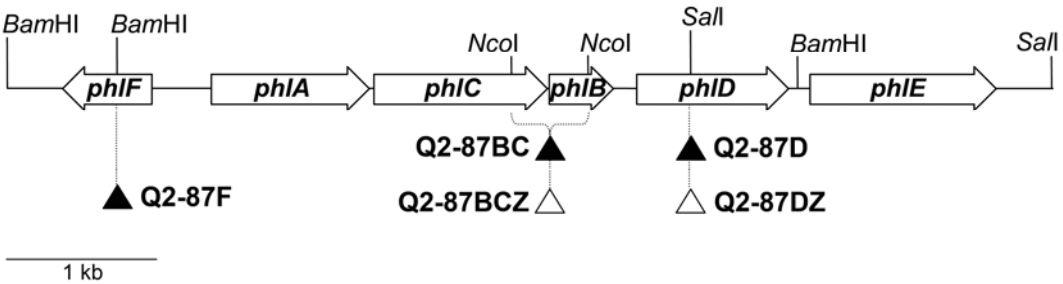

Fig. 1. Physical map of the DNA region encoding genes involved in production of 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* Q2-87. The position and orientation of individual genes are shown by arrows. For gene replacement mutants Q2-87F, Q2-87D, Q2-87BC, Q2-87BCZ, and Q2-87DZ, the black and white triangles indicate sites of insertions of *tet* and *lacZYA* cassettes, respectively.

The antibiotic was dissolved in methanol (1 mg ml^{-1}) and then further diluted with sterile water. All treatments received the same amount of methanol. Five days prior to challenge inoculation (see below), 13 ml of the antibiotic solution was slowly injected with a syringe and needle into the mix in each pot. Controls consisted of methanol and water or water alone injected into the soil. In some experiments, an SAR treatment was included as a control. For this purpose, three lower leaves of a seedling were infiltrated with 10 mM $MgSO_4$ containing avirulent *P. syringae* pv. *tomato* (*avrRpt2*) at 10^7 CFU ml^{-1}, using a syringe without a needle (45). All treatments were arranged in a completely randomized design and replicated 20 to 24 times. Each pot served as a treatment replicate. All experiments were performed at least twice with similar results.

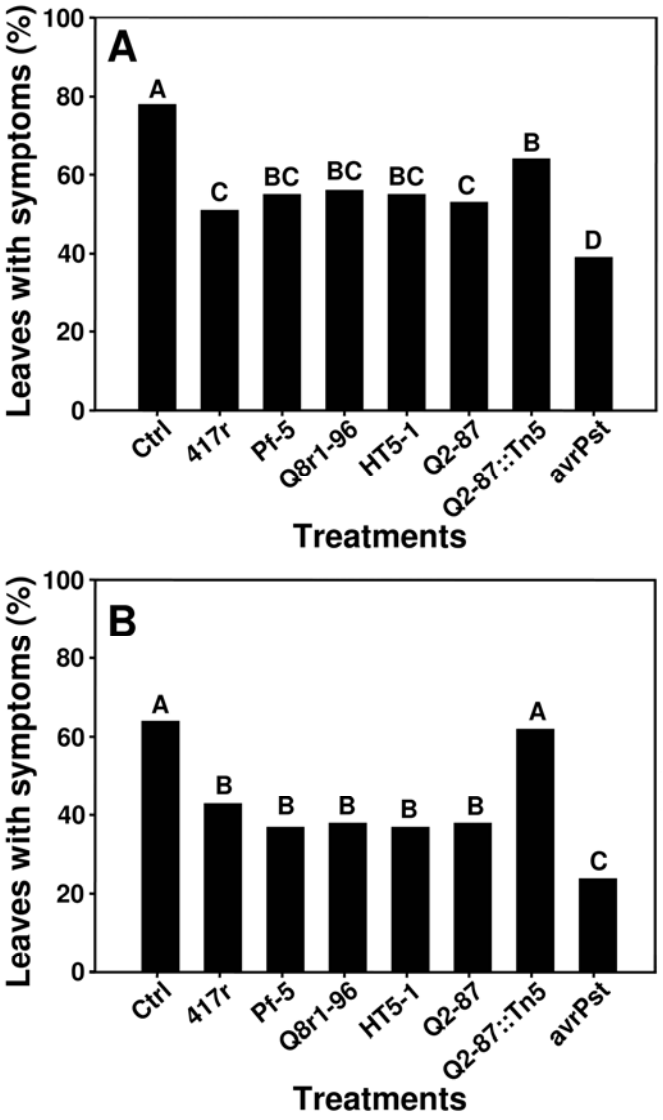

Fig. 2. Induction of systemic resistance in *Arabidopsis thaliana* Col-0 by 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* strains (Pf-5, genotype A; Q2-87, genotype B; Q8r1-96, genotype D; and HT5-1, genotype N), antibiotic-deficient mutant Q2-87::Tn*5*-1, and *P. fluorescens* WCS417r. Two-week-old seedlings were transplanted into a potting soil mix with either 10 mM $MgSO_4$ (Ctrl = nontreated control) or 5×10^7 bacterial cells/g of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *P. syringae* pv. *tomato* (1×10^7 CFU ml^{-1}). For the systemic acquired resistance treatment, three lower leaves of a seedling were infiltrated before challenge with 10 mM $MgSO_4$ containing avirulent *P. syringae* pv. *tomato* (*avrRpt2*) at 10^7CFU ml^{-1}, using a syringe without a needle. Disease severity was evaluated 3 days after challenge inoculation and is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. **A and B**, Two separate experiments. Within each panel, treatments with the same letter are not significantly different (P = 0.05) according to the Fisher's protected least significant difference test.

Challenge inoculation and disease assessment. Plants were subjected to challenge inoculation 3 weeks after being transplanted. One day before challenge, the plants were subjected to 100% relative humidity by placing lids on the trays holding the pots, and then kept at 100% relative humidity after inoculation. Challenge inoculation involved dipping the leaves into a suspension of virulent *P. syringae* pv. *tomato* (1.0, 1.5, or 2.5×10^7 CFU ml^{-1}, depending on the experiment) in 10 µM $MgSO_4$ supplemented with 0.01% of the surfactant Silwet L-77. The concentration of *P. syringae* pv. *tomato* was varied among experiments to alter the severity of disease. The percentage of leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored 3 or 4 days after challenge inoculation (45).

Root colonization by strains of *P. fluorescens*. At the end of each experiment, the roots of four to six plants per treatment were individually harvested and assayed for the population densities of the introduced bacteria. Each pot was cut open along the sides and the soil mix and roots were carefully removed from the pot. The mix was gently teased apart, leaving only the *Arabidopsis* roots with tightly adhering soil mix. The roots were severed from the shoots and the entire root system with adhering mix was weighed (fresh root weight) and placed in a 50-ml screw-cap centrifuge tube with 20 ml of 10 mM $MgSO_4$ and 1.25 g of glass beads (0.6 mm in diameter). Tubes were vigorously agitated with a Vortex mixer on high speed, resulting in the roots being fragmented into small pieces. A 1-ml aliquot was removed from each tube and serially diluted, and then 0.1-ml aliquots of each dilution were spread plated in duplicate on plates of KB agar supplemented with ampicillin (40 µg ml^{-1}), chloramphenicol (13 µg ml^{-1}); and cycloheximide (100 µg ml^{-1}), and rifampicin (75 µg ml^{-1}) (KB^{++++}).

To assess the stability of pMON5122 in the 2,4-DAPG-minus mutants, dilutions of samples containing Q2-87DZ(pMON5122) or Q2-87BCZ(pMON5122) were also plated on KB^{++++} and KB, each supplemented with tetracycline (25 µg ml^{-1}). Samples containing strains Q2-87DZ(pPHL5122) or Q2-87BCZ(pPHL5122) were also plated on KB supplemented with cycloheximide (100 µg ml^{-1}), rifampicin (75 µg ml^{-1}), and kanamycin (50 µg ml^{-1}) or cycloheximide (100 µg ml^{-1}) and kanamycin (50 µg ml^{-1}).

Data analysis. Data of percentage of diseased leaves and bacterial population densities were arcsine and log transformed, respectively, and subjected to standard analysis of variance followed by Fisher's protected least significant difference test (P = 0.05) using STATISTIX (version 8.0; Analytical Software, St. Paul, MN).

RESULTS

Suppression of bacterial speck on Col-0 by 2,4-DAPG-producing *P. fluorescens*. 2,4-DAPG-producing wild-type strains Pf-5 (genotype A), Q2-87 (genotype B), Q8r1-96 (genotype D), and HR5-1 (genotype N) applied as a soil treatment consistently and significantly (P = 0.05) reduced the percentage of diseased leaves on *A. thaliana* Col-0 challenged with *P. syringae* pv. *tomato* compared with the noninoculated control. In two separate experiments, the four 2,4-DAPG producers did not differ significantly(P = 0.001) (Fig. 2A and B) in ability to reduce disease and were equivalent in disease suppression to strain WCS417r, previously shown to be able to induce resistance in *A. thaliana*. The introduced 2.4-DAPG producers were isolated from the roots (see below) but not from the leaves (data not shown), suggesting no direct effect of the bacteria on the pathogen but an induction of systemic resistance.

Avirulent *P. syringae* pv. *tomato* infiltrated into the lower leaves (SAR control) consistently reduced the percentage of diseased

leaves to a significantly greater extent (P = 0.001) than the rhizobacteria applied to the soil (Fig. 2A and B). Throughout the course of this study, the total amount of bacterial speck developing on the Col-0 *Arabidopsis* plants varied among experiments, and was 78 to 40% of the leaves of noninduced control plants with symptoms. Within this range of disease, the ability of the bacterial treatments to suppress the disease was not affected by the total amount of disease that developed in each experiment.

Population densities of the five wild-type pseudomonads in the *Arabidopsis* rhizosphere differed significantly in some experiments (Table 2); however, no strain consistently had a significantly greater or lower density than any other strain (Table 2). For example in experiment 1, Q8r1-96 had a significantly (P = 0.001) higher density than Q2-87 but, in experiment 2, the opposite occurred. In all experiments, population densities were always >log 6.5 CFU g^{-1} root (Table 2), a population sufficient to induce resistance (48).

Effect of loss of 2,4-DAPG production on disease suppression in Col-0 by Q2-87 mutants. All *phlD* or *phlCB* mutants of Q2-87, constructed by transposon mutagenesis (Q2-87::Tn*5*-1) (4) or gene replacement (Q2-87BC, Q2-87BCZ, Q2-87D, and Q2-87DZ), were deficient in 2,4-DAPG production in vitro, as previously described for Q2-87::Tn*5*-1 (4) and demonstrated in this study for Q2-87BC, Q2-87BCZ, Q2-87D, and Q2-87DZ using HPLC analysis (7) (data not shown). All mutants except Q2-87D were used to determine the role of the antibiotic in suppression of *P. syringae* pv. *tomato* in *Arabidopsis*.

In nine separate experiments conducted over a 30-month period of time (five shown here), *Arabidopsis* plants colonized by antibiotic-deficient mutants (Q2-87::Tn*5*-1, Q2-87BC, Q2-87BCZ, or Q2-87DZ) consistently showed significantly (P = 0.001) more disease than plants treated with Q2-87; the amount of disease was either equivalent to that on nontreated control plants or intermediate between the amount of disease on control and Q2-87 treated plants (Figs. 2A and B, 3A and B, and 4A). *phlD* and *phlCB* mutants performed similarly in experiments where they were tested together.

phlF is a regulatory gene in the 2,4-DAPG biosynthetic locus (4) and its disruptions does not result in a loss of 2,4-DAPG production but, rather, an increase. *Arabidopsis* plants treated with Q2-87F had significantly less disease (P = 0.001) than nontreated control or Q2-87::Tn*5*-1- or Q2-87BC-treated plants (Fig. 4A). Q2-87F and Q2-87 did not differ significantly in ability to suppress disease.

Rhizosphere population densities of the antibiotic-deficient mutants tested for ISR activity (Q2-87::Tn*5*-1, Q2-87BC, Q2-87BCZ, and Q2-87DZ) were >log 6.2 CFU g^{-1} of root in all experiments and did not consistently differ from Q2-87 in ability to colonize the rhizosphere (Table 2; experiments 1 to 6). For example, Q2-87 reached a significantly higher density than Q2-87::Tn*5*-1 in experiment 1 (P = 0.001) and experiment 2 (P = 0.002); there was no difference in experiment 3 (P = 0.932); and the density of Q2-87::Tn*5*-1 was significantly (P = 0.003) higher than that of Q2-87 in experiment 4 (Table 2).

Effect of genetic complementation of mutants on disease suppression. The antibiotic-deficient mutants Q2-87DZ and Q2-87BCZ were genetically complemented with the plasmid pMON5122 or pPHL5122, generating the strains Q2-87DZ (pMON5122), Q2-87BCZ(pMON5122), Q2-87DZ(pPHL5122), and Q2-87BCZ(pPHL5122), respectively, and restoring antibiotic production in the mutants. HPLC analysis demonstrated that mutants complemented with pMON5122 and pPHL5122 produced approximately five times more and four times less antibiotic than Q2-87 in broth cultures (data not shown).

Arabidopsis treated with strains Q2-87BCZ(pMON5122) or Q2-87BCZ(pPHL5122) had significantly (P = 0.001) less disease than plants treated with no bacteria, Q2-87BC, or Q2-87BCZ but an equivalent amount of disease to plants treated with Q2-87 (Fig. 3B). Plants treated with Q2-87DZ(pPHL5122) had significantly (P = 0.001) less disease than nontreated plants, Q2-87DZ, or Q2-87::Tn*5*-1, and equivalent disease to plants treated with Q2-87. Plants treated with Q2-87DZ(pMON5122) had significantly (P = 0.001) less disease than nontreated plants, and an equivalent amount of disease to plants treated with Q2-87, Q2-87DZ, or Q2-87::Tn*5*-1 (Fig. 3A). In another experiment (data not shown), plants treated with Q2-87DZ(pMON5122) had significantly less disease than plants treated with the antibiotic-minus mutant.

Rhizosphere population densities of the four genetically complemented mutants were >log 6.3 in all experiments and did not differ significantly from the density of Q2-87 (Table 2). Samples containing mutants complemented with either pPHL5122 or pMON5122 were also plated on media containing kanamycin or tetracycline, respectively. The difference in counts on media with and without kanamycin or tetracycline was used to determine the frequency of cells that retained the plasmids after 3 weeks of growth in the rhizosphere. pPHL5122 was highly stable in the mutants because the counts on media with and without kanamycin were identical. In addition, all colonies transferred from media without kanamycin to media with the antibiotic grew (Table 2;

TABLE 2. Population sizes of introduced wild-type, 2,4-diacetylphloroglucinol mutants and genetically complemented strains in the rhizosphere of *Arabidopsis thaliana* Col-0[y]

	Log CFU/g of root					
Strain	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
WCS417r	7.03 a	6.80 bc	7.08 a	6.59 a	7.46 a	7.25 a
Q8r1-96	7.04 a	6.65 c	6.99 a	…	…	…
HT5-1	6.83 ab	6.68 bc	…	…	…	…
Pf-5	6.84 ab	7.11 a	…	…	…	…
Q2-87	6.77 b	6.87 b	7.01 a	6.04 c	6.68 b	6.83 abc
Q2-87::Tn*5*-1	6.43 c	6.61 c	7.03 a	6.33 b	7.01 ab	…
Q2-87DZ	…	…	…	…	7.70 b	…
Q2-87BC	…	…	…	6.28 b	…	6.95 ab
Q2-87BCZ	…	…	…	…	…	7.14 a
Q2-87DZ(pMON5122)	…	…	…	…	6.62 b (11.5%)[z]	…
Q2-87DZ(pPHL5122)	…	…	…	…	6.69 b (100%)[z]	…
Q2-87BCZ(pMON5122)	…	…	…	…	…	6.37 c (26.4%)[z]
Q2-87BCZ(pPHL5122)	…	…	…	…	…	6.63 bc (100%)[z]
Q2-87F	…	…	…	6.21 bc	…	
P =	0.0009	0.0016	0.3918	0.0034	0.0211	0.00182

[y] Means in the same column followed by the same letter are not significantly different (P = 0.05) according to Fisher's protected least significant difference test. Population sizes of strains in experiments (Exp.) 1, 2, 5, and 6 correspond to strains shown in Figures 2A, 2B, 3A, and 3B, respectively. Data showing the induction of resistance by strains in experiments 3 and 4 are not shown.

[z] Percentage of the cells of the complemented mutants retaining pMON5122 or pPHL5122 when the population densities were determined.

experiments 5 and 6). In contrast, pMON5122 was less stable in Q2-87DZ and Q2-87BCZ. The colony counts of Q2-87DZ(pMON5122) and Q2-87BC(pMON5122) on media with tetracycline were only 11.5 and 26.4%, respectively, of the counts on media without tetracycline. In addition, the same percentages of colonies transferred from media without tetracycline grew on media with the antibiotic (Table 2; experiments 5 and 6).

Effect of addition of pure 2,4-DAPG into soil. All three concentrations of 2,4-DAPG (10, 100, and 250 μM) introduced into the soil mix significantly (P = 0.001) reduced the amount of bacterial speck on plants grown in the treated soil compared with plants grown in the mix treated with water only or water + methanol (Fig. 4B). All concentrations of 2,4-DAPG had equivalent ISR activity. The water + methanol treatment significantly (P = 0.001) reduced disease compared with the water control.

Disease development in NahG *Arabidopsis* and *npr1, jar1,* and *etr1* mutants. To help determine the plant hormone signaling pathway involved in ISR, strain Q2-87 was tested for ISR activity on NahG transgenic *Arabidopsis* and on *npr1-1*, *jar1*, and *etr1* mutant plants. As in earlier experiments, Q2-87 resulted in less bacterial speck on wild-type accession Col-0 compared with the nontreated control (Table 3). Q2-87-treated NahG plants had sig-

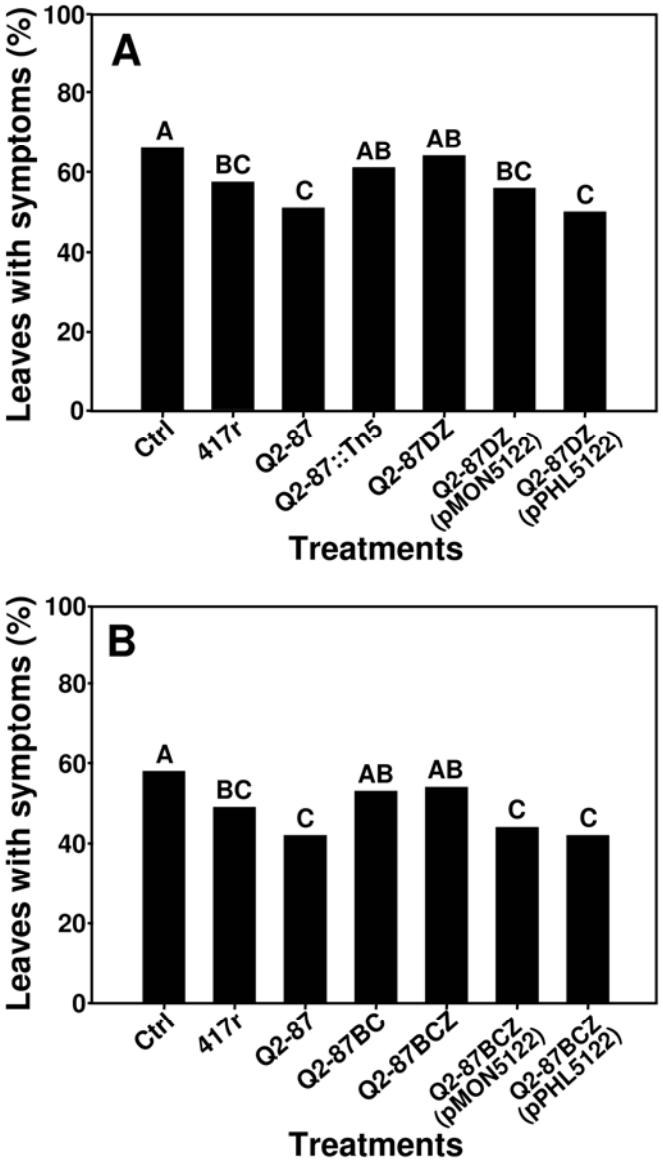

Fig. 3. Induction of systemic resistance in *Arabidopsis thaliana* Col-0 by 2,4-diacetylphloroglucinol-producing strain *Pseudomonas fluorescens* Q2-87 (genotype B), antibiotic-deficient mutants (Q2-87::Tn5-1, Q2-87BC, Q2-87BCZ, and Q2-87DZ), mutants Q2-87BCZ and Q2-87DZ genetically complemented with pPMON5122 or pPHL5122, and *P. fluorescens* WCS417r. Two-week-old seedlings were transplanted into a potting soil mix with either 10 mM $MgSO_4$ (Ctrl = control) or 5×10^7 bacterial cells g^{-1} of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *P. syringae* pv. *tomato* at **A,** 2.5×10^7 or **B,** 1.0×10^7 CFU ml^{-1}. Disease severity was evaluated 3 or 4 days after challenge inoculation and is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. **A and B,** Two separate experiments. Within each panel, treatments with the same letter are not significantly different (P = 0.05) according to the Fisher's protected least significant difference test.

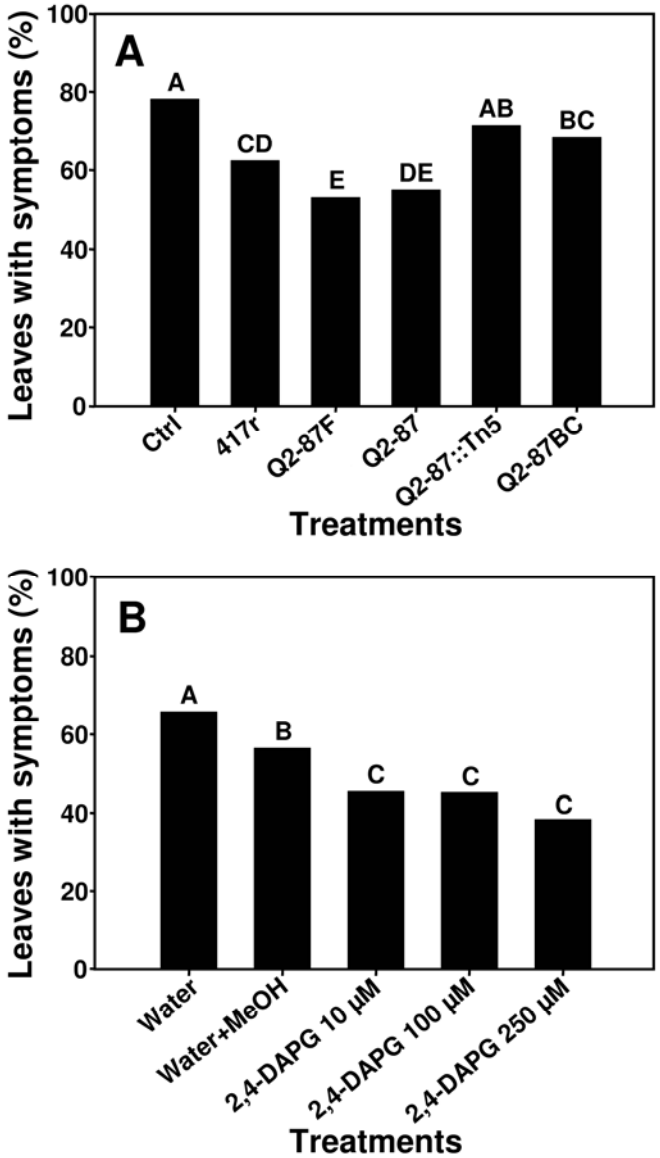

Fig. 4. Induction of systemic resistance in *Arabidopsis thaliana* Col-0 by **A,** 2,4-diacetylphloroglucinol (2,4-DAPG)-producing strain *Pseudomonas fluorescens* Q2-87 (genotype B), antibiotic-deficient mutants (Q2-87::Tn5-1 and Q2-87BC), and regulatory mutant Q2-87F, *P. fluorescens* WCS417r; and **B,** pure 2,4-DAPG applied into the soil (10, 100, and 250 μM). Two-week-old seedlings were transplanted into a potting soil mix with either 10 mM $MgSO_4$ (Ctrl = nontreated control) or bacterial cells at 5×10^7 g^{-1} of soil in 10 mM $MgSO_4$. Plants were grown for another 3 weeks and challenge inoculated by dipping the rosette leaves in a suspension of *P. syringae* pv. *tomato* (1.0×10^7 CFU ml^{-1}). Prior to challenge, 2,4-DAPG dissolved in a methanol-water mix was injected into the soil with a syringe. Equal amounts of methanol-water or water alone were injected as controls (B). Disease severity is expressed as the mean percentage of leaves with necrotic water-soaked symptoms per plant. Treatments with the same letter are not significantly different (P = 0.05) according to the Fisher's protected least significant difference test.

nificantly less disease then nontreated plants. Q2-87 was unable to suppress disease on the *npr1-1*, *jar1*, and *etr1 Arabidopsis* mutants (Table 3), suggesting that the signal transduction pathway was ET and JA dependent.

DISCUSSION

We demonstrated the ability of 2,4-DAPG-producing *P. fluorescens* strains, applied to a potting soil mix, to induce systemic resistance in the leaves of *A. thaliana* against *P. syringae* pv. *tomato*. 2,4-DAPG producers (Pf-5, Q2-87, Q8r1-96, and HT1-5), representing four genotypes (A, B, D, and N) and isolated from three distinct geographic regions of the United States (Texas, Washington State, and Minnesota) were equally effective at inducing resistance. In addition, the 2,4-DAPG producers showed consistent ISR activity over a wide range of disease severity (40% [moderate] to 80% [high] of the leaves with symptoms). We ruled out the possibility of direct interactions between the 2,4-DAPG producers and *P. syringae* pv. *tomato* by showing that the 2,4-DAPG producers did not colonize the leaves and that disease was not suppressed by Q2-87 colonizing the roots of *npr1-1, etr1*, and *jar1* mutants of *A. thaliana* Col-0. We confirmed the ISR assay performance as previously described (45) by including strain WCS417r as a positive control in most of the experiments. In addition, SAR, induced by infiltrating avirulent *P. syringae* pv. *tomato* into the leaves, was included as a control and shown to reduce the amount of disease more than rhizobacteria-mediated ISR, which is characteristic of SAR (26).

Our findings are consistent with those of Iavicoli et al. (21), who demonstrated that the 2,4-DAPG producer CHA0 (genotype A, isolated from a Swiss suppressive soil) induced systemic resistance in *A. thaliana* against *H. arabidopsidis* and implicated 2,4-DAPG as the key determinant of the activity. Given the breadth of the 2,4-DAPG producers now shown to have ISR activity, we think that this trait is common, if not universal, among this important group of biocontrol rhizobacteria.

Previously, Raaijmakers et al. (48) reported that a threshold population density of 10^5 CFU g^{-1} of root was required for induction of resistance in radish by *P. fluorescens* WCS374 against Fusarium wilt caused by *Fusarium oxysporum* f. sp. *raphani*. The same threshold population density was required for direct suppression of *G. graminis* var. *tritici* in the wheat rhizosphere by *P. fluorescens* Q2-87. In the current study, the population densities of the introduced rhizobacteria in the *Arabidopsis* rhizosphere were consistently above 10^6 CFU g^{-1} of root, which is thought to be sufficient to induce resistance (48). Among the wild-type strains tested, rhizosphere population densities differed significantly ($P = 0.05$) within certain experiments but those differences were not consistent across experiments and, thus, were not biologically relevant. The lack of differences in colonization of the *Arabidopsis* rhizosphere, even for Q8r1-96, Pf-5, and Q2-87 (known to differ significantly in colonization of pea and wheat), is not unexpected because the ISR assay utilizes an autoclaved potting soil mix and very high initial doses of rhizobacteria to insure maximum colonization of the roots.

We used a genetic approach, commonly known as "Molecular Koch's Postulates" (35), to determine the role of 2,4-DAPG in the ISR activity of Q2-87. We selected Q2-87 as a representative 2,4-DAPG producer for this portion of the study for several reasons: it is a well-described biocontrol agent (43) with 2,4-DAPG production as the key mechanism of disease suppression (18,63); it is part of ARDRA group 2, which contains 20 of the 22 known genotypes of 2,4-DAPG producers; it is the source of the first 2,4-DAPG biosynthetic locus cloned and sequenced; and it readily produces the antibiotic in the rhizosphere (0.62 ng per 10^5 CFU) (47). Five separate mutants of Q2-87 (Q2-87::Tn*5*-1, Q2-87BC, Q2-87D, Q2-87BCZ, and Q2-87DZ), constructed with different approaches, and with mutations in either *phlD* or spanning *phlCB*, were deficient in 2,4-DAPG production. All four of the mutants selected for testing in the ISR assay consistently resulted in significantly ($P = 0.05$) less control of *P. syringae* pv. *tomato* than Q2-87 when applied to the soil mix. Rhizosphere population densities of the 2,4-DAPG deficient mutants were consistently above 10^6 CFU g^{-1} of root and, although, there were significant ($P = 0.05$) differences among the populations of Q2-87 and the mutants, the differences were not consistent and, thus, not biologically relevant.

Somewhat unexpected was the variability in ISR activity of an individual mutant among the experiments. For example in the experiment shown in Figure 2B, the amount of disease on plants treated with Q2-87::Tn*5*-1 was equivalent to that on the nontreated control plants but, in the experiment shown in Figure 2A, disease severity on plants treated with this mutant was intermediate between that on the noncontrol and Q2-87-treated plants. These results suggest the possibility of a second determinant of ISR associated with Q2-87 that may not be consistently expressed in the rhizosphere. Multiple determinates of ISR are known to be associated with other ISR-inducing rhizobacteria (3,40) and, when more than one determinant is strongly expression in situ, the use of a genetic approach to determine the role of a single determinant can be complicated.

To fulfill Molecular Koch's Postulates, the 2,4-DAPG-deficient mutants Q2-87BCZ and Q2-87DZ were genetically complemented with pMON5122 or pPHL5122, which resulted in four mutants (Q2-87BCZ/pMON5122, Q2-87DZ/pMON5122, Q2-87BCZ/pPHL5122, and Q2-87DZ/pPHL5122) restored in 2,4-DAPG production and concomitantly restored (partially or completely) in ISR activity. Rhizosphere population densities of the mutants were consistently above 10^6 CFU g^{-1} of root. Again, although there were significant ($P = 0.05$) differences in population densities among Q2-87, mutants, and complemented strains, those differences were not consistent.

We were surprised, at first, by the performance of the complemented mutant Q2-87DZ(pMON5122), which produced four times as much 2,4-DAPG as Q2-87 in vitro yet was only partially restored in ISR activity in one of the experiments (disease ratings intermediate between the wild type and Q2-87DZ). This inconsistency may be explained by the rhizosphere population data, which showed that, although the population density was log 6.6 CFU g^{-1} of root for this strain at the end of the experiment, only 11.5% of the cells retained the plasmid and, thus, the ability to produce 2,4-DAPG. Interestingly however, twice as many (26.4%) cells of Q2-87BCZ/pMON5122) retained the plasmid. In contrast, pPHL5122 was very stable in both mutants. The

TABLE 3. Induction of systemic resistance in *Arabidopsis thaliana* Col-0, NahG transgenic, and *npr1-1*, *jar1*, and *etr1* mutants by *Pseudomonas fluorescens* Q2-87[z]

	Leaves with symptoms of bacterial speck (%)									
	Experiment 1		Experiment 2			Experiment3		Experiment 4		
Treatment	NahG	Col-0	NahG	*npr1-1*	Col-0	*npr1-1*	Col-0	*jar1*	*etr1*	Col-0
Nontreated	82.6 a	54.6 a	60.6 a	67.9 a	40.5 a	71.7 b	50.0 a	53.6 a	54.7 a	56.8 a
Q2-87	68.1 b	42.1 b	48.0 b	67.4 a	31.9 b	79.1 a	37.0 b	59.4 a	58.2 a	36.6 b

[z] Means in the same column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

basis for this difference in stability of the two plasmids is not understood.

To complement the findings generated with the completion of Molecular Koch's Postulates, we applied chemically synthesized 2,4-DAPG to the soil in pots with noninoculated plants prior to challenge with *P. syringae* pv. *tomato*. At all three concentrations tested (10, 100, and 250 μM), the antibiotic reduced disease compared with the water control and the water + methanol control and Q2-87, supporting a role for the antibiotic as a determinant of ISR.

In *A. thaliana*, the ISR activity induced by *P. fluorescens* WCS417r operates through a signal transduction pathway that is ET and JA dependent and depends on NPR1 (46). To begin to understand whether Q2-87-induced activity functions through a pathway similar to that induced by WCS417r, we tested Q2-87 on transgenic NahG plants and *npr1-1*, *etr1*, and *jar1* mutants. Q2-87 induced resistance on NahG plants, indicating that ISR is not SA dependent, but Q2-87 had no ISR activity on *npr1-1*, *etr1*, and *jar1* plants. Collectively, these results suggest a pathway similar to that induced by WCS417r. Iavicoli et al. (21) reported that CHA0-induced ISR in *A. thaliana* against *H. arabidopsidis* functioned through the ET- and JA-dependent pathway. However, WCS417r and CHA0 showed differential responses on *etr*1-1 *and ein*2-1 mutants, indicating some differences in the pathways. On the *etr1* mutant, Q2-87 behaved similarly to WCS417r. Most surprisingly, however, Iavicoli et al. (21) reported that the application of pure 2,4-DAPG induced resistance in *npr*1-1 and *jar*1-1, suggesting that the antibiotic induces resistance through a different pathway than CHA0. Further research with a wider collection of *Arabidopsis* mutants is needed to fully elucidate whether the steps in the signal transduction pathway activated by Q2-87 are identical to the steps induced by WCS417r or CHA0, or if they diverge in some way from the steps induced by these two strains. Future work also will focus on this question, and on the activity of the pure antibiotic on ISR.

It is now clear that some plant species select and support specific populations of 2,4-DAPG producers as a method to defend themselves against certain soilborne pathogens (64). The best examples of this are the take-all decline (TAD) soils in Washington State and The Netherlands (12,49), the black root rot-suppressive soils in Switzerland (51,65), and the Fusarium wilt-suppressive soil in Mt. Vernon, WA (30). In these and other soils, studies have focused on the direct antagonism of the 2,4-DAPG producers on certain target pathogens. Our current study and those of Iavicoli et al. (21) and Siddiqui and Shaukat (56) strongly suggest a wider role for 2,4-DAPG producers in enhancing the basal resistance to foliar and root pathogens in plants growing in natural and agricultural ecosystems. This hypothesis is supported by the following: (i) certain crop species and genotypes of 2,4-DAPG producers show a mutual preference for each other in the rhizosphere (29–31); (ii) 2,4-DAPG producers occur on roots at population densities above the threshold (10^5 CFU g^{-1} of root) necessary for induction of resistance, especially when the plant is grown in monoculture (29,31,47); and (iii) 2,4-DAPG has been isolated from the rhizosphere of several plant species (5,47) grown in natural soil. Studies are currently under way to determine the breadth of activity of 2,4-DAPG-based suppressiveness in TAD soils against a broad range of foliar and root pathogens.

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