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Induced defence in tobacco by *Pseudomonas chlororaphis* strain O6 involves at least the ethylene pathway

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Abstract

The antifungal phenazines produced by certain root colonizing pseudomonads are credited with contributing to the biocontrol activity of these isolates. However, the phenazine producer *Pseudomonas chlororaphis* O6 also induces systemic resistance in tobacco. Protection against two foliar bacterial pathogens, the wild fire pathogen, *Pseudomonas syringae* pv. *tabaci*, and *Erwinia carotovora* subsp. *carotovora* that causes a soft rot, was observed. A mutation in the bacterial global regulator *gacS* gene eliminated certain secondary traits, including phenazine production, but did not prevent induction of resistance in tobacco to *P. syringae* pv. *tabaci*. However, induction of resistance to *E. carotovora* subsp. *carotovora* was impaired in plants colonized by the GacS mutant compared to the level of protection in plants colonized by wild-type *P. chlororaphis* O6. Colonization by both the wild-type and the GacS mutant caused increased accumulation for transcripts of plant defense genes regulated by ethylene. Tobacco transformed with a mutant *etr1* gene to reduce ethylene sensitivity did not display induced resistance to *E. carotovora* when colonized by *P. chlororaphis* O6. We conclude that inducers other than phenazines appear to initiate the pathways for systemic resistance induced by *P. chlororaphis* O6.

Keywords: *Erwinia carotovora* subsp. *carotovora*; Ethylene; GacS; Induced resistance; Phenazines; Plant defense signaling; *Pseudomonas chlororaphis* O6; *Pseudomonas syringae* pv. *tabaci*

1. Introduction

Biological control of plant pathogens by microbes has several bases. The production of antimicrobial compounds that deter growth of the pathogen is one of the mechanisms. Phenazines produced by several plant-associated pseudomonads, *Pseudomonas chlororaphis*, formerly *aureofaciens*, 30–84 [23], *P. fluorescens* 2–79 [29], and *P. chlororaphis* P2E and O6 [16,26], inhibit the growth of fungi, including plant pathogens. Field studies indicate that the phenazines are produced when the bacteria colonize plant roots and that they are important in biological control [28,30].

A second method of control of pathogens is by the induction of plant defenses. Differential expression of defense genes is controlled through two pathways, the salicylic acid (SA) or the jasmonic acid (JA)/ethylene pathway [6,13]. Enhanced expression from the promoter of the PR-1 gene, associated with the SA pathway, is observed in plants with root colonization by bacilli and pseudomonads [22,37]. In contrast, the induced systemic resistance (ISR) caused by colonization by a *P. fluorescens* isolate is SA-independent but requires the JA/ethylene pathway [25].

We are interested in the interactions between plant roots and the pseudomonad isolate, *P. chlororaphis* O6. The *P. chlororaphis* O6 bacterium appears to be similar to *P. chlororaphis* 30–84 in producing the same phenazines with their characteristic orange pigmentation [26]. The *P. chlororaphis* O6 strain, isolated from the roots of wheat grown in Logan, UT, was identified from its fatty acid composition conducted by MIDI Inc., Newark, DE. Because phenazines are redox active, it seemed possible that their

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production might stimulate resistance in plant tissues [33]. Indeed while we were conducting the studies reported in this paper, Audenaert et al. [1] published that phenazine-1-carboxylate and a siderophore, pyochelin, produced by *Pseudomonas aeruginosa* 7NSK2 both contributed to the ability of this isolate to induce systemic resistance in tomato.

The studies in this paper compare the response of tobacco to the wild-type *P. chlororaphis* O6 strain and a GacS mutant, a mutant lacking production of phenazines but still secreting siderophore. Previous studies with *P. chlororaphis* 30–84 showed that the GacA–GacS two-component sensor-kinase system is a key regulator of such traits as the production of phenazines, extracellular protease and hydrogen cyanide [2,11,24]. GacA/GacS systems also regulate the production of antifungal compounds and/or virulence factors in other plant-associated pseudomonads [5,14]. We examined systemic induction of defense in plants with roots colonized by *P. chlororaphis* O6 or its GacS mutant by determining whether the symptoms of disease caused by two leaf pathogens, *Pseudomonas syringae* and *Erwinia carotovora*, were altered. To probe which pathways of induced resistance were activated, we used transformed tobacco altered in ethylene perception or in the accumulation of SA to determine whether resistance induction by *P. chlororaphis* O6 or the GacS mutant was modified. Also, we assayed the transcript levels of defense genes that were typical of the JA/ethylene or the SA pathways of induced resistance. We find resistance induced by *P. chlororaphis* O6 was not correlated with phenazine formation but was dependent upon GacS when tobacco was challenged with *E. carotovora*.

2. Materials and methods

2.1. Bacterial growth medium

The *P. chlororaphis* O6 strain was stored in 20% glycerol (v/v) at −80 °C. Wild-type *P. chlororaphis* O6 and its derivatives were grown with the following media: potato dextrose (PD, Difco, Detroit, MI, USA), King's medium B (KB) (Difco, Detroit, MI, USA) or minimal medium with glucose as the carbon source [15]. Agar was used in these plates at 2% (w/v) and kanamcyin (Km) was added at 20 μg ml^{-1} when the GacS mutant was grown and Km plus tetracycline (Tc) at 20 μg ml^{-1} for growth of the complemented mutants. Broth cultures were shaken at 100 rpm at 28 °C.

2.2. Construction of a GacS mutant of P. chlororaphis O6

Wild-type *P. chlororaphis* O6 was subjected to Tn-5 mutagenesis using a Tn-Lux cassette constructed by Wolk et al. [34] and kanamycin-resistant isolates were selected that no longer produced the characteristic orange color of the *P. chlororaphis* O6-phenazines when grown on KB plus kanamycin. Restoration of phenazine formation was achieved by transfer to one of the transposon mutants of plasmid, pOKB6.3, a broad host range plasmid pCPP46 (Tc^R) containing a 6.3 kb *Bam* HI–*Kpn* I fragment from wild-type *P. chlororaphis* O6 bearing the *gacS* gene. GacS mutants were constructed in *P. chlororaphis* O6 by replacement of its wild-type gene with a disrupted *gacS* gene, using a method previously described [19]. Briefly, the *gacS* gene from *P. chlororaphis* O6 was disrupted by insertion of a 900 bp kanamycin resistance gene, digested with *Eco* RI from pRL648 [8] into the unique *Eco* RI site of the *gacS* ORF between predicted amino acids (aa) 287 and 288. The mutated gene was inserted into the plasmid eviction vector pCPP54 [19] and was transferred to *P. chlororaphis* O6 by triparental mating. Marker-exchange mutants were isolated on selective medium [19] and mutation of the *gacS* gene was verified by Southern hybridization using pOKB6.3 as the probe labelled with digoxigenin following manufacture's instructions (Roche, Indianapolis, IN, USA). Complementation of the mutants was achieved using plasmid pOKB6.3.

2.3. Characterization of the GacS mutant cells

Isolation and detection of extracellular protease activity involved measurement of the zone of clearing around colonies plated onto medium containing 2% skim milk and agar after 3 days of incubation at 28 °C [2]. Siderophore production was determined on chromazol-S-plates by measuring the size of the decolorized halo around the inoculation site after 3 days of incubation at 28 °C [27]. Inhibition of the growth of *Rhizoctonia solani*, isolate RE1, a pathogen of sugar beet [18], a carrot pathogen, *Sclerotium rolfsii* and the cause of crown rot of wheat *Fusarium culmorum* [16] was determined by streaking the bacterial isolate on potato dextrose agar plates, 4 cm away from a 1 cm cube plug of agar excised from the growing edge of a culture of the fungus. No bacteria were inoculated in the controls. The growth of the fungus was measured after three days of incubation at 28 °C.

2.4. Induction of plant disease resistance by O6 and GacS mutant: whole plant studies

Transgenic NahG tobacco and its parental line cv. Xanthi-nc were provided by Dr John Ryals, Novartis, Research Triangle Park, NC, USA. Wild-type plants GTX3 were obtained from Dr L-H Zhang, Institute of Molecular Agrobiology, The National University of Singapore, Singapore. Tetr18 and its parental line cv. Samsun-NN were obtained from Dr Huub JM Linthorst, Leiden University, Leiden, Netherlands. Tobacco seeds were surface-disinfested by soaking in 70% ethanol for one to 2 min followed by soaking in 1% sodium hypochlorite for 30 min. Seeds were rinsed in sterile distilled water three

times. The sterilized tobacco seeds were placed on MS agar (GIBCO-BRL, Rockville, MD, USA); with the addition of agar 0.8%, and in some studies sucrose to 1.5%), in microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Seeds were germinated in growth chambers with a 14 h light period at 25–28 °C and were transferred under sterile conditions to sterilized microtiter plates. Two weeks after transplanting, suspensions containing 10^{6-7} colony forming units (cfu) of *P. chlororaphis* O6 or the GacS mutant were pipetted into the agar to contact the roots. These bacterial cells had been grown for 14 h to stationary-phase in KB medium, pelleted by centrifugation at 10,000*g*, and washed once with sterile water. Other treatments were: water as a control, 1 mM salicylic acid (Sigma, St Louis, USA), or 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC) (Sigma, St Louis, USA). Also, as a control for systemic stimulation of the SA pathway of defense, the seedlings were sprayed with a mixture of 1 mM SA. The seedlings were grown for an additional 7–10 days when leaf tissue for Northern analysis was harvested and immediately frozen in liquid nitrogen. Other leaves were ground in sterile water and plated onto KB and KB-Km plates to check for the presence of *P. chlororaphis* O6 and the GacS mutant, respectively. Colonization of the roots by *P. chlororaphis* O6 and the GacS mutant was assessed by transfer of the seedling roots onto KB medium for *P. chlororaphis* O6 growth and KB-Km plates for the growth of the GacS mutant.

To examine for the stimulation of plant defense gene expression, leaves were ground into a powder in liquid nitrogen and stored at −70 °C. The powdered leaf material was extracted for RNA using Tri reagent according to the manufacturer's protocol (MRC, Cincinnati, OH, USA). Northern analysis was performed to determine the abundance of transcripts from plant defense genes by following standard methods [36]. Total RNA (10 μg lane^{-1}) was separated on 1.5% formaldehyde agarose gels and transferred to positively-charged nylon membranes. Membranes were hybridized at 65 °C overnight with digoxigenin-labelled RNA probes (Roche, Indianapolis, IN, USA). After high stringent washing at 65 °C, immunological detection of bound probes was performed according to the standard protocol for CDP-Star™ (Roche, Indianapolis, IN, USA). The genes examined for induction were: *PR-1a*, encoding a pathogenesis related protein 1a, *PR-1g*, encoding a pathogenesis related protein 1g, *LOX,* encoding lipoxygenase, and *HMGR*, encoding 3-hydroxy-3-methyl-glutaryl-CoA reductase [35,36]. PR-1a is associated with the SA pathway of defense, whereas PR-1g, LOX and HMGR are linked with the ethylene and/or jasmonate signaling [4,9,12].

To determine whether plants were protected against disease, plants were challenged with a pathogen 1 week after the wild-type or GacS bacterial inoculation or after chemical treatments. The seedlings were challenged with *E. carotovora* subsp. *carotovora* strain SCC1 or *P. syringae* pv. *tabaci*, applied as 20 μl droplets of suspensions (10^{8-9} cfu ml^{-1}) to the leaves. Disease was assessed after 24 h for *E. carotovora* and 7 days for *P. syringae* pv. *tabaci.* The 0–10 disease scales were established with the following guides: for *P. syringae* pv. *tabaci*: 0, no symptoms; 2, mild yellowing on inoculated leaf; 5, extensive yellowing and brown necrotic lesions; 6–9, intensification of leaf necrosis; 10, whole plant release; for *E. carotovora*: 0, no symptoms; 2, mild yellowing on inoculated leaf; 5, partial softening collapse of leaf at the inoculated site; 6–9, intensification of leaf soft rot; 10, leaves rotted and whole plant collapse. These scales were established using inoculum with increasing cfu ml^{-1}.

In studies of disease protection in more mature plants, the sterile-grown seedlings were transferred to commercial potting mix (Garden Grow Co., Independence, OR, USA). Three weeks after transfer, plants were inoculated by drenching the potting mix till run off with a suspension (10^7 cfu ml^{-1}) of *P. chlororaphis* O6 or GacS mutant cells. After 7 days plants were challenged by infiltration of segments of the leaves with suspensions at 1×10^6 and 1×10^7 cfu ml^{-1}. Plants were grown for further 4–8 days in a growth chamber to allow symptoms to develop. Colonization of the roots by *P. chlororaphis* O6 or by the GacS mutant was checked by transfer of about 3 cm lengths of the intact root, including the root tip, to KB or KB-Km medium. Growth of colonies was assessed visually after 4 days of incubation at 28 °C. Colonization of the leaves was examined by grinding 1 cm discs of leaf tissue in sterile water and plating onto KB or KB-rif (25 μg ml^{-1}) medium.

2.5. Suspension-cultured cell studies

N. tabacum L. cv. Xanthi tobacco suspension cells were cultured in the dark at 25 °C in MS medium supplemented with 1 mg l^{-1} α-naphthaleneacetic acid, 0.1 mg l^{-1} benzyladenine and 3% (w/v) sucrose. Cells were subcultured weekly and used 4 days after subculture [35,36]. The plant cell suspensions were inoculated with 10^7 cfu 50 ml^{-1} of *P. chlororaphis* O6 or GacS mutant cells that had been grown to stationary-phase in KB, pelleted by centrifugation and washed once with water prior to resuspension in water and immediate use. Controls were treated with equal volumes of sterile water. At the time prior to inoculation, and after 24 h, plant cells were harvested, RNA extracted and Northern analysis performed as described for the leaf tissues.

3. Results

3.1. Cloning of the gacS gene

Plasmid pOKB6.3, containing a 6.3 kb *Bam*HI–*Kpn*I fragment from *P. chlororaphis* O6, restored the ability of an uncharacterized Tn5 mutant of *P. chlororaphis* O6 to produce phenazines. This fragment contained the *gacS* gene

from *P. chlororaphis* O6 which had a predicted 2754 bp open reading frame (GenBank accession number AF192795) encoding a peptide of 918 amino acids with molecular mass 101 kDa and pI 5.3. The deduced amino acid sequence of the *P. chlororaphis* O6 *gacS* gene had high identity with other GacS kinases from *P. chlororaphis* 30–84 (97%), *P. fluorescens* (91%), *P. tolassi* (87%), and *P. syringae* pv *syringae* (78%). The predicted GacS protein from *P. chlororaphis* O6 had all the conserved domains found in other sensor kinases, as discussed by Heeb and Haas [11]. Two transmembrane domains existed in the N-terminal region and a conserved histidine residue, corresponding to the H-box, was located at amino acid 294. Two linker domains, proposed to be involved in the process of kinase activation were present. The conserved aspartate–aspartate (DD), the aspartate (D), and the lysine (K) residues of characteristics of receiver domains were found in the C-terminal regions of the deduced GacS amino acid sequence.

3.2. Characterization of GacS mutants of *P. chlororaphis O6*

Four GacS mutants of *P. chlororaphis* O6, M1–M4, were derived by insertion of a kanamycin-resistance gene at the *Eco* RI site within the *gacS* gene of wild-type *P. chlororaphis* O6. Although the wild-type when grown on KB medium produced three phenazines, which migrated on thin layer chromatography plates with the same R_f values as those in extracts from *Pc* 30–84 [23,26], no phenazines were detected from the GacS mutants (Table 1). The GacS mutants lacked production of extracellular protease (Table 1). They overproduced siderophore (Table 1). Each GacS mutant lacked the ability to inhibit growth of the pathogenic fungi, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium culmorum* that was observed with the wild-type strain (data not shown). All of these properties were restored in a stable manner to at least wild-type level by complementation with the wild-type *P. chlororaphis* O6 gene from plasmid pOBK63 (Table 1). Further studies were conducted with mutant M1 and its complemented strain C1.

Table 1
Phenotypes of wild-type, GacS and complemented GacS mutants of O6

Strains[a]	Production of secondary metabolites		Production of siderophore (mm)[b]
	Phenazines[c]	Protease (mm)[d]	
O6	+	7–8	4–5
M1–M4	–	0	7–8
C1–C4	+	7–8	5–6

[a] Each study was repeated at least three times with the wild-type *P. chlororaphis* O6, the GacS mutants, M1–M4, and the complemented GacS mutants, C1–C4 strains.
[b] Siderophore was assessed on chromazol-S plates by measuring the size of the decolorized halo in mm around the inoculation site after 3 days of incubation at 28°C.
[c] The three phenazines were detected by TLC analysis and by spectrophotometric analysis for peak absorbance wavelength [26].
[d] Protease activity was detected on skim milk agar plates and denoted as the zones of clearing in millimeters.

3.3. Colonization of tobacco roots and induction of defense mechanisms

P. chlororaphis O6 aggressively colonized roots of tobacco seedlings under sterile and non-sterile conditions in potting mixes containing a mixture of other bacteria (detected at $2.3–7.1 \times 10^6$ cfu g^{-1} matrix) and fungi, including a parasitic *Trichoderma* species. Characteristic orange colonies of *P. chlororaphis* O6 were detected growing from the roots when these were placed onto KB medium. Fluorescent kanamycin-resistant colonies of the GacS mutant were recovered from the plants inoculated with the GacS mutant. In other studies, where cell counts were made by dilution plating, GacS cells were recovered at the same level as the wild-type cells (data not shown). Microbial growth from roots grown in non-sterile soils is shown in Fig. 1. The figure shows orange coloration typical of *P. chlororaphis* O6 when rhizosphere soil and roots from *P. chlororaphis* O6-inoculated plants are plated onto KB-rif medium. Roots from plants not inoculated with *P. chlororaphis* O6 do not show this coloration and fungi grew profusely, as shown in Fig. 1. Roots from tobacco grown under sterile and non-sterile conditions 1 or 2 weeks after inoculation with GacS always produced colonies that

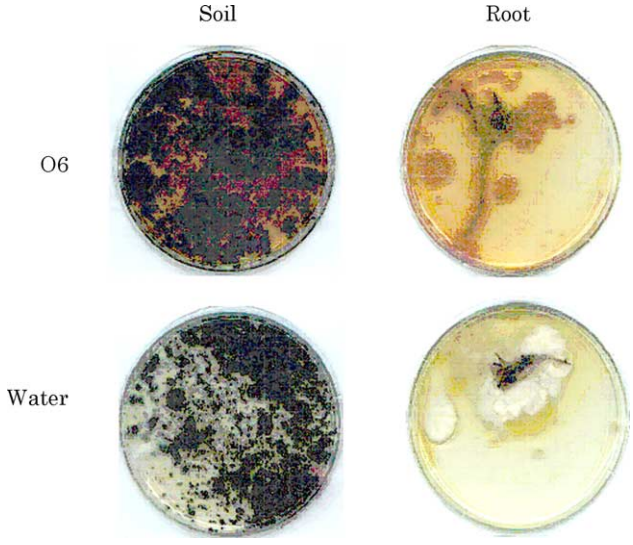

Fig. 1. Aggressive colonization of plant roots in a non-sterile soil by *P. chlororaphis* O6. Tobacco seedlings grown from seed with or without *P. chlororaphis* O6 inoculum for 4 weeks under sterile conditions were transplanted into non-sterile potting soil. After 1 week, roots and adhering soil were transferred to KB medium plates that were incubated at 27 °C for 4 days. Orange-pigmented colonies characteristic of *P. chlororaphis* O6 are present on the soil plate and are associated with the root of the *P. chlororaphis* O6-inoculated plant. Other bacteria and fungi are present in the soil and are associated with the roots of the control plates. Fungi are not visible from the roots of the *P. chlororaphis* O6-colonized root, indicating control of fungal growth. This finding was observed in five different studies.

were fluorescent and rifampin- and kanamycin-resistant when cultured on KB plates (data not shown). Rifampin- and kanamcycin-resistant fluorescent colonies were not observed from the control roots not inoculated with GacS.

3.4. O6 and the gacS mutant induce accumulation of defense gene transcripts

Hybridization of RNA extracts from leaves of plants with roots colonized by *P. chlororaphis* O6, showed increased accumulation of transcripts hybridizing to probes for the tobacco defense genes *PR-1g*, *HMGR* and *LOX* (Fig. 2(A)). No transcript accumulation was observed with the *PR-1a* probe, although hybridization was observed in extracts from plants sprayed with salicylic acid (Fig. 2(A)). Also, no increase in GUS activity was observed after *P. chlororaphis* O6 colonization of seedlings of tobacco engineered to express GUS from a *PR-1a* promoter fusion (data not shown).

To verify that the changes in RNA levels were a result of the inoculation of the plants with *P. chlororaphis* O6 and not due to a contaminating organism, we examined the response in tobacco suspension cells. Exposure of suspension-cultured tobacco cells to *P. chlororaphis* O6 induced increased transcript accumulation of *PR-1g* and *HMGR*, but neither *LOX* nor *PR-1a* (Fig. 2(B)).

Root colonization by cells of the GacS mutant likewise caused increased accumulation of the same defense gene transcripts as the wild-type in seedlings. Exposure of the suspension-cultured tobacco cells to cells of the GacS mutant resulted in an increase of transcripts from the *PR-1g* and *HMGR* genes (Fig. 2(A) and (B)).

3.5. Induction of systemic resistance by O6 and the gacS mutant

Symptom development in seedling tobacco grown in otherwise sterile conditions confirmed that *P. chlororaphis* O6 provided protection against pathogen challenge. The results of challenges of wild-type and transgenic tobacco seedlings with two pathogens, the soft rot pathogen *E. carotovora* subsp. *carotovora* and the wild-fire pathogen *P. syringae* pv. *tabaci* are shown in Table 2. *P. chlororaphis* O6 colonization resulted in protection of the plant to both pathogens in two wild-type tobacco cultivars, Xanthi-nc, and Samsun-NN. However, the responses to *Erwinia* and the pseudomonad differed for plants colonized by GacS. In the wild-type plants, the GacS mutant protected at the same level as the wild-type against the wild fire pathogen but the GacS mutant was ineffective against the soft rot pathogen.

To initiate understanding of the pathways involved in resistance, we examined the responses in plants treated with SA or ACC and in transformed plants deficient in SA-mediated resistance, because of the presence of a NahG gene, and in an ethylene-insensitive line (Table 2). The results suggested to us the involvement of several factors in the induced protection. Applications to wild-type Xanthi of SA and of ACC to Samsun-NN increased resistance to both pathogens. Loss of the SA-pathway in the NahG-tobacco eliminated the protection against both pathogens when SA was applied. However, in the NahG plants colonized by *P. chlororaphis* O6, the GacS mutant or the complemented mutant protection was evident against *P. syringae*. With challenge by *E. carotovora*, some protection was observed in the NahG-plants colonized by the wild-type *P. chlororaphis* O6, the complemented mutant and also the GacS mutant. Loss of the ethylene pathway in the transformed tobacco eliminated induced resistance to the soft rot pathogen by ACC treatments and in the plants colonized by *P. chlororaphis* O6, GacS and the complemented GacS mutant. However, the ethylene-insensitive transformed tobacco was still protected against *P. syringae* challenge when the roots were colonized by wild-type, GacS mutant and complemented strains.

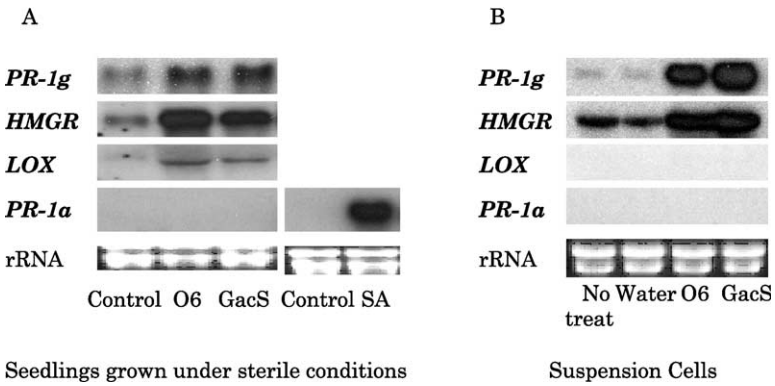

Fig. 2. *P. chlororaphis* O6 and GacS induced accumulation of defense gene transcripts. (A) Seedlings were grown on sterile medium and were harvested at 5 weeks, 1 week after inoculation with *P. chlororaphis* O6 or GacS cells or treatment with water as non-inoculated controls. Other plants were treated for 24 h with 1 mM salicylic acid to induce *PR-1a* expression. (B) Suspension cultured cells, 3 days after transfer into new medium, were inoculated with *P. chlororaphis* O6 or GacS cells. Other suspensions were treated with water as a control. Plant cells were harvested after 24 h. RNA was prepared from leaf and suspension cells and probed for transcript accumulation for specific genes as described in Section 2. Data shown are representative of three separate studies.

Table 2
Protection by *P. chlororaphis* O6, GacS and the complemented GacS mutant against disease in tobacco caused by two leaf pathogens

	Disease index: tobacco cultivar			
	Xanthi-nc		Samsun-NN	
	Wild-type	NahG-10	Wild-type	Tetr18
(A) Pathogen: Erwinia carotovora subsp. carotovora				
Control	8.00	8.67	8.11	8.50
O6	4.50*	4.33*	4.33*	7.29
GacS	8.00	5.83*	6.89	8.00
Compl GacS	4.17*	3.67*	4.44*	8.00
ACC	ND	ND	5.50*	8.83
SA	5.50*	8.50	ND	ND
LSD 0.05	1.66	2.27	1.85	1.32
(B) Pathogen: Pseudomonas syringae pv. tabaci				
Control	8.50	7.83	7.67	7.17
O6	2.17*	2.00*	3.67*	1.33*
GacS	2.00*	4.01*	3.68*	2.67*
Compl GacS	2.83*	4.88*	3.00*	1.50*
ACC	ND	ND	4.00*	8.83
SA	2.33*	6.50	ND	ND
LSD 0.05	2.02	1.84	1.46	1.54

Data are based on inoculations of four leaves of 12 3-week-old seedlings grown in microtiter plates containing sucrose-amended MS medium. Plants were treated with *P. chlororaphis* O6 and its mutants or with the chemicals SA and ACC 2 weeks after planting as described in Section 2. Disease index is based on a scale of 1–10. Asterisk means significantly different from control at LSD ($P = 0.05$).

Disease symptoms in potting soil-grown mature plants challenged with the wild-fire pathogen *P. syringae* pv. *tabaci* were reduced in severity by *P. chlororaphis* O6-root colonization. There was strong consistent protection against *P. syringae* when inoculated at 10^6 cfu ml^{-1} (Fig. 3). There was no protection at the higher inoculum level of 10^7 cfu ml^{-1}. Rifampicin-resistant, fluorescent cells of the pathogen *P. syringae*, but not the orange-colored cells characteristic of *P. chlororaphis* O6, were recovered from ground extracts of the leaf tissues. No fluorescent bacterial cells were recovered from non-challenged leaves. With plants colonized by GacS, protection against *P. syringae* was variable: protection, as determined by reduction in the leaf symptoms, equivalent to that generated by colonization with the wild-type *P. chlororaphis* O6 was observed in two out of five studies. An example of the lesser level of protection in GacS-colonized plants observed in the other studies is provided in Fig. 3.

4. Discussion

The GacS protein of the two-component GacA–GacS sensor system regulated the production of phenazines, protease and siderophore by *P. chlororaphis* O6, showing similarity to *P. chlororaphis* 30–84. Like *P. chlororaphis* 30–84 [3], the *gacS* lesion did not prevent root colonization by the bacterium under sterile and the non-sterile laboratory greenhouse conditions that we used. The *P. chlororaphis* O6 wild-type isolate was very aggressive in its colonization being readily culturable from roots even although the potting soil had high counts for other culturable organisms. This aggressive colonization of *P. chlororaphis* O6 under non-sterile conditions with an array of other soil organisms was observed also for bean and wheat (data not shown).

The *P. chlororaphis* O6 wild-type isolate stimulated systemic resistance in tobacco. Resistance to two types of leaf pathogens, a soft rot pathogen and one that caused necrosis through the production of toxins, was observed in seedlings grown under sterile conditions. Protection against the wild fire pathogen was demonstrated also in more mature plants transferred to grow in non-sterile potting soil.

Exposure of suspension-cultured tobacco cells and the seedlings to *P. chlororaphis* O6 and the GacS mutant stimulated accumulation of transcripts of plant defense genes under ethylene and JA regulation, *PR-1g* [9], and *HMGR* [4]. Although transcripts from *LOX* [12] increased in the seedlings, this response was not observed in the suspension cells, perhaps because of the lack of developmental factors required for expression of this gene. These findings suggested that *P. chlororaphis* O6 activated at least the ethylene pathway of plant defense. Unlike *P. putida* [37] and the fluorescent pseudomonads tested by Park and Kloepper [22], there was no stimulation of *PR-1a*

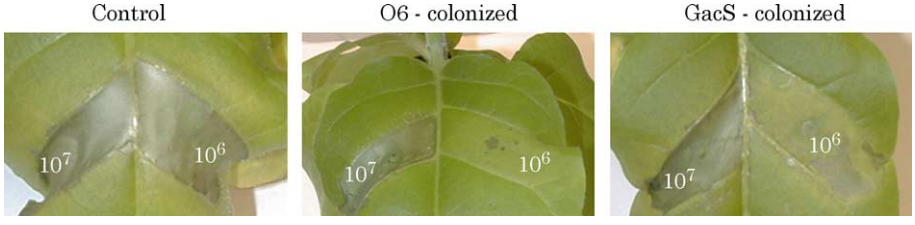

Fig. 3. Reduced symptom formation in mature tobacco leaves 4 days after inoculation with *Pseudomonas syringae* pv. *tabaci*. Seedlings were grown under sterile conditions for 4 weeks prior to transplant into non-sterile potting soil. Inoculum of *P. chlororaphis* O6 or GacS cells was applied at 7 weeks by drenching the soil as described in Section 2. At 8 weeks, leaves were challenged with *Pseudomonas syringae* pv. *tabaci* infiltrated at doses of 10^6 or 10^7 cfu ml^{-1} as indicated. Symptoms were recorded at 4 days after pathogen challenge. The data are from one of five studies where the results of *P. chlororaphis* O6-colonization were consistent. In three out of the five studies, GacS mutant colonization caused no protection. The leaf shown is representative of one of the two studies where symptoms were reduced in GacS mutant-colonized plants. In the other three studies the extent of the symptoms was the same as in control plants not colonized by *P. chlororaphis* O6.

expression indicative of the SA pathway. The protective response induced by *P. chlororaphis* O6 was similar to the effect of *P. fluorescens* WCS417r on *Arabidopsis* [25], where the ethylene-pathway of defense was activated. However, our findings demonstrate the complexity of the induced resistance response.

Treatment of wild-type tobacco with SA and ACC showed that both chemicals invoked resistance to both pathogens. In contrast, studies in *Arabidopsis*, suggest resistance to soft rot *Erwinia* pathogen involves the ethylene/JA pathway [21] and resistance to *P. syringae*, the SA-dependent pathway [6]. Our findings with the ethylene-insensitive tobacco line substantiate the involvement of an ethylene-based pathway in *P. chlororaphis* O6-induced resistance for *E. carotovora*. Also, the lack of SA accumulation in NahG-transformed tobacco did not affect the efficacy of resistance initiated by *P. chlororaphis* O6-colonization towards the soft rot pathogen.

Our findings support the concept that resistance against *P. syringae* in tobacco involves different pathways than resistance against *E. carotovora* (Fig. 4). Resistance was not affected by ethylene-insensitivity in the Tetr18 transformed lines. However, the role of an SA-pathway in induced resistance to *P. syringae* is questioned because (1) we failed to observe transcript accumulations for *PR-1a* and (2) resistance in the plants colonized by *P. chlororaphis* O6 mutant and wild-type strains was still apparent in the NahG-transformed plants. The nature of this induced resistance is unknown at present. However, our findings reiterate that different plants may utilize different pathways for defense against a pathogen. Previous studies have observed that resistance of tobacco and bean to the necrotrophic fungus *Botrytis cinerea* required the SA pathway whereas for *Arabidopsis*, the JA/ethylene pathway was more important [7,20,31].

Other findings that that we cannot explain are (1) why the GacS mutant afforded protection against the wild-fire pathogen but not against the soft rot pathogen in tobacco grown under sterile conditions (Fig. 4) and (2) why colonization by the GacS mutant conferred greater protection against the soft rot pathogen in the SA-deficient NahG transformants than the wild-type line. We speculate that *P. chlororaphis* O6 wild-type induced different responses effective against *Erwinia* than those induced by the GacS mutant. Could the overproduction of siderophore by the GacS strain be involved? Another distinguishing feature of the strains is that on KB medium, the wild-type strain grew as tight colonies whereas the mutant formed spreading colonies (data not shown), suggesting that cell surface features vary between mutant and wild-type. The nature of the inducing features from *P. chlororaphis* O6/GacS cells is unknown. Our original hypothesis that the phenazines from *P. chlororaphis* O6 were inducers of plant defense was not supported because GacS as well as *P. chlororaphis* O6 stimulated accumulation of *PR-1g* and *HMGR* transcripts under otherwise sterile conditions and we have never observed phenazine production with the GacS mutant. Thus, our findings differ from the report of Audenaurt et al. [1] implicating phenazine and siderophore as factors that stimulate ISR with a *P. aeruginosa* isolate. However, whether the mutant and wild-type strains differ in other factors that are effectors for plant defense stimulation, flagellins [10] and lipolysaccharide [17,32], has not been studied.

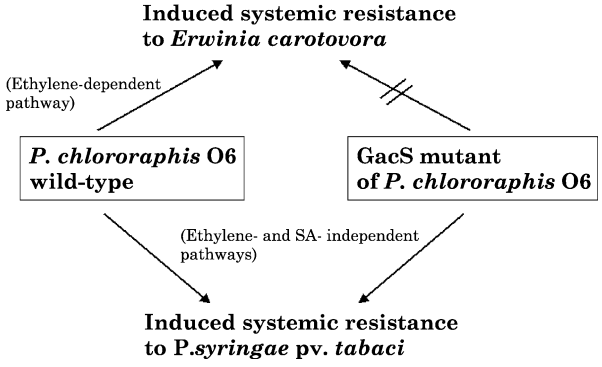

Fig. 4. Summary scheme for defense pathways against two pathogenic bacteria initiated in tobacco by root colonization by the wild-type *P. chlororaphis* O6 and its GacS mutant.

Protection against *P. syringae* in mature plants colonized by the GacS mutant was more variable than that observed with colonization by the wild-type. We speculate that other rhizosphere organisms could be causing this variability. For instance, we observed amongst the competing rhizosphere bacteria *Bacillus* isolates that degraded the acyl homoserine lactones produced by the *P. chlororaphis* O6 strains (data not shown). If traits that are involved in initiation of resistance are regulated through the acyl homoserine lactone system [2,3], then, because the GacS strain makes acyl homoserine lactones in less quantity than the wild type (data not shown), the GacS mutant cell-interaction with the plant might be more sensitive to the affects of the *Bacillus* species.

In conclusion, we report that root colonization by *P. chlororaphis* O6 stimulated ISR to two leaf pathogens, although by apparently different pathways. An ethylene-signaling pathway was needed for protection against the soft rot pathogen whereas ethylene- and SA-independent pathways, of as yet uncharacterized nature, was involved for the induced resistance to the wild fire pathogen. The global regulator protein kinase, GacS, in *P. chlororaphis* O6, was required for protection against the soft rot pathogen but not for induction of resistance to the wild-fire pathogen.

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