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FULL RESEARCH PAPER

Tobacco cultivars vary in induction of systemic resistance against *Cucumber mosaic virus* and growth promotion by *Pseudomonas chlororaphis* O6 and its *gacS* mutant

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Abstract The colonization of plant roots with certain rhizosphere bacteria promotes plant growth and induces long lasting systemic protection against a broad spectrum of plant pathogens. The role of the global regulator, GacS, in the rhizosphere colonist *Pseudomonas chlororaphis* O6 in stimulating growth promotion and induced resistance against *Cucumber mosaic virus* was examined in tobacco. Responses were compared in tobacco cvs Samsun and GX3. Root colonization of Samsun with wild-type O6 and the *gacS* complemented mutant-elicited reduced viral symptoms and viral titre. On GX3, there was little affect on symptoms when roots were colonized by the wild-type, *gacS* mutant or complemented mutant but colonization by both the wild-type and the *gacS* mutant lowered viral titre. Wild-type O6 and the *gacS* mutant caused plant growth to be maintained in both tobacco cultivars after viral infection, although the affect was stronger with GX3 than Samsun. In contrast, although a chemical inducer, benzothiadiazole, reduced symptoms and viral titre in both cultivars, plant growth was suppressed. Our results indicate rhizobacteria-elicited induced viral resistance without a negative impact on growth but there was a differential response between cultivars. Detailed knowledge regarding the mechanisms inherent to these differences between cultivars requires further investigation.

Keywords *Cucumber mosaic virus* · GacS two component system · Induced systemic resistance · Plant growth promotion

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Introduction

Certain root-associated bacteria (rhizobacteria) enhance plant productivity (Kloepper et al. 1980). Plant growth promotion by rhizobacteria can involve production of various plant growth regulators, including indole-acetic acid (IAA) (Prinsen et al. 1993), gibberellic acid (Glick1995), brassinosteroid-like compounds (Jung et al. 2002) and cytokinins (Timmusk et al. 1999). Another mechanism for growth promotion is reduction in ethylene levels due to

microbial expression of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick 1995). Solubilization of phosphates by rhizobacteria can improve the uptake of phosphate (Glick 1995; Kim et al. 2002), thereby leading to increased plant growth. Certain volatile metabolites, including 2,3-butanediol and acetoin, produced by root-colonizing microbes also enhance plant growth (Han et al. 2006; Ryu et al. 2003).

In addition to promoting plant growth, rhizobacteria can influence pathogens by direct or indirect effects. Indirectly, root-colonization by certain rhizobacteria induces systemic resistance that is effective against viral, bacterial, and fungal pathogens, as well as nematodes. For instance, the plant growth-promoting rhizobacteria (PGPR) strains *Bacillus pumilus* SE34 and *Serratia marcesans* 90–166 elicited induced systemic resistance (ISR) on five and six plant species, respectively (Kloepper and Ryu 2006). The term, ISR, describes ''activation of the host plant's physical or chemical defenses by an inducing agent'' (Kloepper et al. 1992). The criterion used to define ISR is to observe separation on the plant between the site of pathogen attack and the colonization site for the inducing bacteria. ISR occurs in many plants including carnation, cucumber, tobacco, tomato, bean, radish, and *Arabidopsis* (Van Loon et al. 1998).

In contrast to signalling pathways for resistance induced by necrotic pathogens or by certain chemicals (Ryals et al. 1996), ISR does not require a salicylic acid-dependent pathway (Pieterse et al. 1996, 2002; Ryu et al. 2004b; Van Loon et al. 1998). Several microbial determinants have been associated with elicitation of ISR; 2,4-diacetylphloroglucinol (Iavicoli et al. 2003), the O-antigen from lipopolysaccharide (Van Peer and Schippers 1992), and the volatile organic compound, 2,3-butanediol (Han et al. 2006; Ryu et al. 2004a).

Colonization of tobacco roots by *Pseudomonas chlororaphis* O6 elicits ISR against soft rot caused by *Erwinia carotovora* subsp. *carotovora*, and wildfire caused by *Pseudomonas syringae* pv. tabaci (Spencer et al. 2003). Cucumber plants colonized with *P. chlororaphis* O6 are protected against target leaf spot caused by *Corynespora cassicola* (Kim et al. 2004). The global regulator, GacS, in *P. chlororaphis* O6 was required for the production of bacterial determinants related to ISR, including phenazines and 2R,3R-butanediol (Han et al. 2006; Kang et al. 2007; Spencer et al. 2003). Interestingly, GacS in strain O6 negatively regulates the production of the growth-promoting regulator, IAA (Kang et al. 2006).

Induced resistance is a valuable strategy for control of plant pathogens, due to its long-lasting effects against a broad range of pathogens. Chemical elicitors including salicylic acid, β-aminobutyric acid (BABA), and benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH, Actigard^{TM}) also exist (Vallad and Goodman 2004). However, although BTH induces the production of high levels of the defence-related proteins, chitinase, peroxidase, and glucanase, there is a reduction in growth in *Arabidopsis* (Dietrich et al. 2005). Under field conditions, the spray application of the chemical elicitor BTH to wheat reduces crop yields in the absence of pathogens (Heil and Baldwin 2002; Heil et al. 2000). This phenomenon was explained by considering that there is a fitness cost to produce defence-related metabolites in the absence of pathogen attack (Heil et al. 2000).

In this study, our main objective was determine whether root colonization by *P. chlororaphis* O6 would elicit ISR effective against *Cucumber mosaic virus* (CMV). We investigated whether a global regulator, GacS, in *P. chlororaphis* O6 was required for any observed ISR; in tobacco, the *gacS* mutant of O6 failed to induce resistance to *E. carotovora* whereas ISR was induced for the wildfire pathogen (Spencer et al. 2003). We used tobacco cvs Samsun and GX3, to determine whether there was a cultivar affect. Additionally we examined how the wild-type, *gacS* mutant and complemented strains affected plant growth in the two cultivars. We found root colonization affected growth promotion and ISR differentially depending on the tobacco cultivar.

Materials and methods

Bacterial strains and plasmids

The *gacS* mutant and its complemented mutant were generated from wild-type *P. chlororaphis* O6, as described in Spencer et al. (2003). The strains were grown in Pseudomonas Agar F (Difco, Detroit, MI, USA), King's medium B (KB) broth, or Luria Bertani (LB) broth supplemented with 20 μg ml^{-1} of kanamcyin (Km) for growth of the *gacS* mutant and

20 μg ml^{-1} of Km and tetracycline (Tc) for the growth of the complemented mutant. Broth cultures were shaken at 100 rpm at 28°C. For long-term maintenance, the bacterial strains were preserved in LB broth containing 15% glycerol (v/v) at −70°C.

Bacterial and chemical pre-treatment

Seeds of tobacco (*Nicotiana tabacum*) cvs Samsun NN and GX3 were surface-disinfested by soaking in 70% ethanol for 2 min, followed by 30 min in 1% sodium hypochlorite. The seeds were rinsed thoroughly in sterile distilled water (SDW) and transferred to commercial potting mix, Speedling Mix (Speedling,. Inc., Sun City, FL, USA) without sterilization. Three weeks after transfer, plants were inoculated by drenching the potting mix with 20 ml of a suspension (10^{8-9} cfu ml^{-1}) of *P. chlororaphis* O6 wild-type, *gacS* mutant, or complemented mutant cells. These bacterial cells had been grown for 36 h to stationary-phase in KB broth, pelleted by centrifugation at 10,000g, and washed once with SDW. Other treatments applied to the roots of the three week-old plants included water as a control, or 1 mM benzothiadiazole (BTH) (Syngenta, NC, USA) in order to induce the salicylic acid-dependent signalling pathway for induced resistance (Ryals et al. 1996). The seedlings were grown for an additional 7 days after the root treatments prior to viral challenge or the assessment of leaf and root colonization by the pseudomonad mutant and wild-type strains.

To examine colonization of tobacco by the wild type O6 strain, the natural resistance of the wild-type to rifampicin was used as a selective tool for growth of these cells by supplementation of growth medium with this antibiotic at 20 μg ml^{-1}. Leaves were ground in SDW and plated onto KB-rifampicin agar to determine the presence of *P. chlororaphis* O6 cells. The colonization of the roots was assessed by the transfer of seedling roots onto KB-rifampicin agar and observing orange-coloured cells growing from the root surface.

Cucumber mosaic virus challenge and enzyme-linked immunosorbent assay of viral titre

Leaf inoculation of CMV was performed as previously described in Ryu et al. (2004b). Detection of CMV in systemic tobacco leaves was determined by the antigen-coated plate, indirect enzyme-linked immunosorbent assay (ELISA), as previously described by Garcia-Ruiz and Murphy (2001). Each treatment included eight replications with one plant per replication. Water treatments applied to the tobacco plant were used as controls. A mock treatment included a non-virus treated plant. The whole study was repeated. Data shown are for one study because both showed similar results.

Evaluation of plant growth

To evaluate the promotion of plant growth, the total leaf surface area, shoot fresh weight, and stem diameter (crown part) of the tobacco plants were measured 21 days after inoculation or mock treatment, as described by Ryu et al. (2004b). The experiment was repeated twice with eight replications, with one plant per replication.

Data analysis

Data from each experiment were analyzed using JMP software (SAS institute, Cary, NC). The significance of bacterial treatment effects were determined by the magnitude of the F value ($P = 0.05$). When a significant F test was obtained, separation of means was accomplished by Fisher's protected least significant difference.

Results

Effect of bacterial root colonization on CMV infection

The leaves of both tobacco cultivars showed mosaic and chlorotic symptoms after infection by CMV (Fig. 1: cv. Samsun). Eventually, the leaflets became elongated and narrow, developing a characteristic shoestring morphology as the infection grew more severe with time.

Inoculation of the roots of Samsun and GX3 with the wild-type strain of *P. chlororaphis* O6 produced roots that were colonized throughout their length but no colonization of the shoots was demonstrated. With Samsun tobacco, the typical CMV symptoms, as measured by the visual rating system (number of symptomatic leaves per plant), were reduced when

Fig. 1 Images of CMV-inoculated tobacco cv. Samsun grown without (left image, control) and with (right image, O6) colonization of roots by *P. chlororaphis* O6

the plants had roots colonized by *P. chlororaphis* O6 (Fig. 2A). The observed reduction in symptoms was similar in extent to that observed in the BTH-treated plants (Fig. 2A). Likewise, viral titres were reduced in Samsun treated with *P. chlororaphis* O6 in comparison with the control plants. Root colonization by the *gacS* mutant did not elicit a significant ISR or lowered titre of CMV in Samsun tobacco. A high level of protection, as determined by reduced symptoms and viral titres, was restored when the plants were colonized by the complemented *gacS* mutant (Fig. 2A and B). BTH treatments produced a high level of reduction in symptoms and viral titre although this was accompanied by reduced growth (Table 1).

With GX3, root colonization by *P. chlororaphis* O6 did not elicit a significant reduction in viral symptoms although viral titre was significantly reduced (Fig. 3A and B). Results were similar when the plants were colonized by the *gacS* mutant or the wild-type strain (Fig. 3 A and B). Colonization with the complemented *gacS* mutant also had no significant effects on either viral symptoms or viral titres. Thus, ISR was not elicited in GX3 to the same level as in Samsun tobacco. BTH treatment was, however, as effective in reducing viral symptoms and titre in GX3 as in cv. Samsun (Fig. 3A and 3B).

Effect of *P. chlororaphis* O6 on the growth of two CMV-infected tobacco cultivars

Three growth parameters were evaluated at the end of the experiments (21 days post-CMV inoculation dpi): total leaf surface area, shoot fresh weight of

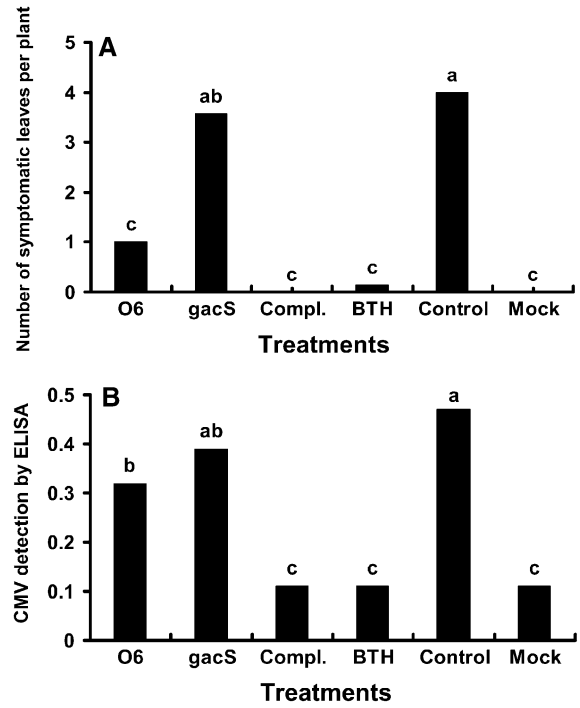

Fig. 2 Systemic resistance against CMV in the tobacco cv. Samsun induced by root colonization with strains of *P. chlororaphis* O6. Disease estimations were taken three weeks after CMV inoculation, which occurred seven days after root treatment of four week-old tobacco with bacterial strains: wild-type (O6), *gacS* mutant (gacS), complemented *gacS* mutant (compl.) or 1 mM BTH or water. Mock plants were grown without root colonization by *P. chlororaphis* O6 and had no inoculation with CMV. Control plants were grown without root colonization by *P. chlororaphis* O6 but were challenged with CMV. (**A**) Number of symptomatic leaves per plant 21 days after CMV inoculation. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$. (**B**) Virus titres (relative units) measured by ELISA 21 days after CMV inoculation of the tobacco plants. Each treatment included eight replications with one plant per replication. The studies were repeated twice. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$

above ground tissue, and stem diameter on both tobacco cultivars. CMV infection of Samsun had no effect on fresh weight and stem diameter but leaf surface area was decreased (Table 1). Although symptoms were reduced in Samsun by root colonization with wild-type *P. chlororaphis* O6, total leaf surface area and stem diameter were greater than for the CMV-challenged plants lacking O6-root colonization (Table 1). Root colonization with the *gacS* mutant and the complemented mutant gave similar

Table 1 Effect of *Pseudomonas chlororaphis* O6 treatment on growth of tobacco cv. Samsun three weeks after CMV inoculation

Treatment	Plant growth parameters[a]		
	SFW (g)	TLSA (cm^2)[b]	SD (mm)
O6	18.6bc	74.2c	6.0c
GacS	19.3bc	71.5c	5.7bc
Compl-*gacS*	22.3c	80.9c	6.1c
1 mM BTH	5.1a	39.2a	4.1a
Water control	16.2b	56.8b	5.2b
Mock (No CMV)	19.5bc	81.8c	5.3b

[a] Each treatment included eight replications with one plant per replication. The experiment was repeated twice with similar results. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$

[b] Total leaf surface area was measured for the 7th and 8th leaves from the top of each plant. Abbreviations: TLSA; total leaf surface area, SFW; shoot fresh weight, and SD; stem diameter. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$

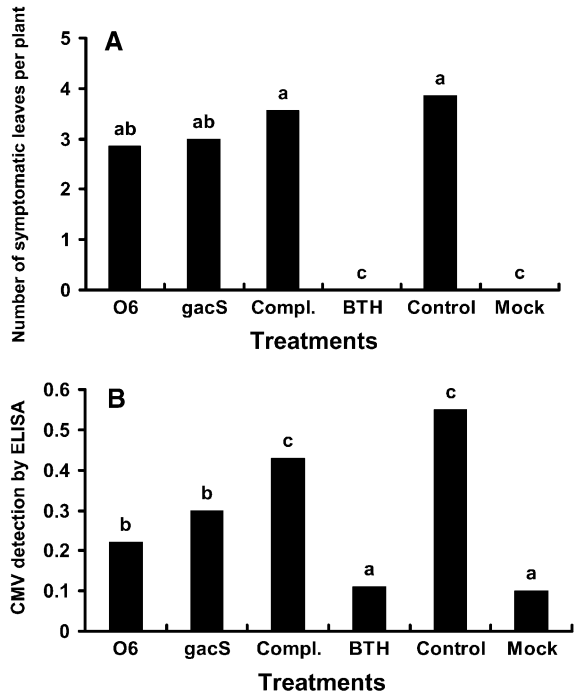

Fig. 3 Systemic resistance against CMV in the tobacco cv. GX3 induced by root colonization with strains of *P. chlororaphis* O6. Disease estimations were taken three weeks after CMV inoculation, which occurred seven days after root treatment of four week-old tobacco with bacterial strains: wild-type (O6), *gacS* mutant (gacS), complemented *gacS* mutant (compl.) or 1 mM BTH or water. Mock plants were grown without root colonization by *P. chlororaphis* and had no inoculation with CMV. Control plants were not colonized by *P. chlororaphis* O6 but were challenged by CMV. (**A**) Number of symptomatic leaves per plant 21 days after CMV inoculation. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$. (**B**) Virus titres (relative units) measured by ELISA 21 days after CMV inoculation of tobacco plant that pre-treated strain O6. Each treatment included eight replications with one plant per replication. The studies were repeated twice. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$

results. Thus, ISR eliminated some of the growth effects caused by viral infection in Samsun. Drench application of 1 mM BTH greatly reduced both the symptoms of the CMV infection and plant growth in both cultivars.

In GX3 tobacco, CMV infection reduced shoot fresh weight and total leaf surface area, but stem diameter was not reduced by viral infection (Table 2). Root colonization with the wild-type, the *gacS* mutant and the complemented *gacS* mutant restored growth as measured by all three parameters in the viral-infected plants; growth surpassed that observed in the non-CMV challenged plants without O6-root colonization.

Discussion

This work extended previous studies where other plant growth-promoting rhizobacteria (PGPR) have been shown to induce systemic resistance against CMV (Murphy et al. 2003; Raupach et al. 1996; Ryu et al. 2004b). Employing the same assay system, we observed that the rhizobacterium *P. chlororaphis* strain O6 generated ISR to reduce viral titres of CMV although the extent of the response differed between the two tobacco cultivars tested. Notably reduction in viral symptoms was more apparent in Samsun than GX3 tobacco. A functional *gacS* gene in the root colonizer *P. chlororaphis* O6 was required to reduce viral titre in cv. Samsun but not GX3. Our findings show that the disease assessment tool, in this case viral leaf symptoms versus viral titre levels, may result in different conclusions as to the efficacy of ISR induced by a rhizosphere bacterium. The factors that influence these two indices of viral disease are not known.

Table 2 Effect of *Pseudomonas chlororaphis* O6 treatment on plant growth of tobaccocv. GX3 at three weeks after CMV inoculation

Treatment	Plant growth parameters[a]		
	SFW (g)	TLSA (cm^2)[b]	SD (mm)
O6	18.3b	109.5c	6.4b
GacS	23.5c	115.8c	7.7c
Compl-*gacS*	22.8c	114.3c	8.0c
1 mM BTH	8.8a	61.7a	4.8a
Water control	9.3a	57.1a	4.6a
Mock (No CMV)	15.2b	81.8b	5.2a

[a] Each treatment included eight replications with one plant per replication. The experiment was repeated twice with similar results

[b] Total leaf surface area was measured for the 7th and 8th leaves from the top of each plant. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$. Abbreviations: TLSA; total leaf surface area, SFW; shoot fresh weight, and SD; stem diameter. Different letters indicate significant differences between treatments according to LSD at $P = 0.05$

The nature of the components that are regulated by *gacS* in *P. chlororaphis* O6 and are involved in inducing viral resistance in Samsun is currently unknown. Previously, Maurhofer et al. (1998) showed that *P. fluorescens* strain CHA0 significantly reduced symptom development (number of lesions and lesion diameter) caused by *Tobacco necrosis virus* (TNV) in tobacco. The *gacA* mutant of this pseudomonad elicited ISR to TNV at the wild-type level, showing the process was independent of the bacterial production of hydrogen cyanide, 2, 4-diacetylphloroglucinol, and pyoluteorin. Whether resistance to TNV and CMV requires similar triggers is not currently known. GacS in *P. chlororaphis* O6 positively regulated phenazine, 2,3-butanediol, and hydrogen cyanide production but negatively regulated IAA synthesis (Han et al. 2006; Kang et al. 2006, 2007; Spencer et al. 2003).

We found that rhizosphere colonization affected the growth of the CMV-infected plants, with growth in cv. GX3 being promoted more than with cv. Samsun. There was more growth protection and less apparent ISR against CMV infection in GX3. However, root colonization of *P. chlororaphis* O6 greatly induced resistance against CMV, but less growth promotion in Samsun. It seems that there is a trade-off between plant growth promotion and induced systemic resistance against CMV infection in these two cultivars following *P. chlororaphis* O6 colonization. As there was no effect of the presence or absence of GacS in the root-colonizing pseudomonad on growth promotion, we cannot correlate improved growth with 2R,3R-butanediol production or IAA levels by the root colonizer. Although butanediol promoted tobacco and *Arabidopsis* growth (Han et al. 2006; Ryu et al. 2003; 2004a), its synthesis in *P. chlororaphis* O6 required GacS (Han et al. 2006). In contrast, GacS repressed IAA production in *P. chlororaphis* O6 (Kang et al. 2006), a finding that differs from the results of transformation of *Enterobacter cloacae*, where a plasmid-borne *gacS* gene enhanced IAA synthesis (Saleh and Glick 2001).

We confirmed that ISR induced with BTH was accompanied by reduction in plant growth, supporting the allocation fitness cost concept of Heil et al. (2000). However, reduced plant growth was not apparent for ISR induced by rhizosphere colonization with *P. chlororaphis* O6. Thus, the use of a bacterial amendment was more advantageous to the plant over the use of a chemical protectant under our test conditions.

In conclusion, *P. chlororaphis* O6 maintained growth of tobacco and limited viral titre by inducing ISR against CMV. The extent of protection as determined by symptom formation was cultivar-dependent. Growth promotion in the presence of the virus was more apparent in GX3 whereas ISR was greater in cv. Samsun. The global regulator, GacS, in *P. chlororaphis* O6 was not required for growth maintenance but was required for ISR in Samsun. Future studies are required to understand at the biochemical and molecular levels the differences between these cultivar responses.

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