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Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against *Cucumber mosaic virus* by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway

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Summary

***Arabidopsis thaliana* ecotype Columbia plants (Col-0) treated with plant growth-promoting rhizobacteria (PGPR) *Serattia marcescens* strain 90-166 and *Bacillus pumilus* strain SE34 had significantly reduced symptom severity by *Cucumber mosaic virus* (CMV). In some cases, CMV accumulation was also significantly reduced in systemically infected leaves. The signal transduction pathway(s) associated with induced resistance against CMV by strain 90-166 was determined using mutant strains and transgenic and mutant *Arabidopsis* lines. NahG plants treated with strains 90-166 and SE34 had reduced symptom severity indicating that the resistance did not require salicylic acid (SA). Strain 90-166 naturally produces SA under iron-limited conditions. Col-0 and NahG plants treated with the SA-deficient mutant, 90-166-1441, had significantly reduced CMV symptom severity with reduced virus accumulation in Col-0 plants. Another PGPR mutant, 90-166-2882, caused reduced disease severity in Col-0 and NahG plants. In a time course study, strain 90-166 reduced virus accumulation at 7 but not at 14 and 21 days post-inoculation (dpi) on the non-inoculated leaves of Col-0 plants. NahG and *npr1-1* plants treated with strain 90-166 had reduced amounts of virus at 7 and 14 dpi but not at 21 dpi. In contrast, no decrease in CMV accumulation occurred in strain 90-166-treated *fad3-2 fad7-2 fad8* plants. These data indicate that the protection of *Arabidopsis* against CMV by strain 90-166 follows a signaling pathway for virus protection that is independent of SA and NPR1, but dependent on jasmonic acid.**

Keywords: plant growth-promoting rhizobacteria, *Cucumber mosaic virus*, induced resistance, salicylic acid, jasmonate.

Introduction

Of the various defense responses that plants elicit against pathogens, one that is highly sophisticated is the gene for gene interaction between the products of a plant-encoded resistance (*R*) gene and its corresponding pathogen-encoded avirulence (*Avr*) gene. This interaction triggers programmed cell death at the site of pathogen attack, a phenomenon referred to as a hypersensitive response (HR). Subsequent to HR, there is enhanced resistance in other leaves on that plant to further attacks by the same or other pathogens. This resistance is called systemic acquired resistance (SAR) (Ryals *et al.*, 1994). SAR typically provides a non-specific and long-lasting induced resistance to a broad spectrum of pathogens. Biological and chemical inducers such as avirulent pathogens or salicylic acid (SA) and its analogues, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) and dichloroisonicotinic acid (INA), also elicit SAR (Ryals *et al.*, 1994). SAR is characterized by the accumulation of SA and pathogenesis-related (PR) proteins such as PR-1, PR-2, and PR-5 in *Arabidopsis* and tobacco (Ryals *et al.*, 1996). These PR genes serve as hallmarks of SAR in several plant species (Delaney, 1997; Delaney *et al.*, 1995; van Loon, 1997). Salicylic acid plays a critical role as a signal molecule that elicits SAR. For example, transgenic salicylate hydroxylase (*nahG*) from *Pseudomonas putida*, which

renders SA inactive by converting it to catechol, blocks SAR in tobacco and *Arabidopsis* and prevents PR gene expression (Delaney, 1997; Delaney *et al.*, 1995; Mauch-Mani and Metraux, 1998).

The mechanisms of induced resistance for some virus–host interactions have been reported. Naylor *et al.* (1998) showed that SA did not prevent the accumulation of *Cucumber mosaic virus* (CMV) in the inoculated leaves of tobacco. However, treatment of tobacco with SA followed by inoculation with CMV resulted in SAR expressed as delayed symptom development, perhaps ascribable to the interference of the systemic movement of CMV.

NPR1 is essential for SAR against bacteria and fungi (Cao *et al.*, 1994, 1998). Wong *et al.* (2002) found that SA and non-lethal concentrations of cyanide and antimycin A (AA) could induce resistance to *Turnip vein clearing virus* (TVCV). The resistance to TVCV elicited by cyanide and AA did not require *NPR1* or *PR-1* genes. These studies indicate that the defense mechanisms divide into two signaling pathways downstream of SA (Murphy *et al.*, 1999). One pathway downstream of SA signaling elicits resistance to bacteria and fungi by *NPR1* and *PR* gene expression and a second pathway from SA induces resistance to virus infection by a plant mitochondrial enzyme, alternative oxidase (AOX) (Murphy *et al.*, 1999).

Induced systemic resistance (ISR) elicited by plant growth-promoting rhizobacteria (PGPR) has shown promise in managing a wide spectrum of plant pathogens, including virus, in several plant species under greenhouse and field environments (Kloepper, 1996; Leeman *et al.*, 1996; Murphy *et al.*, 2000, 2003; Raupach *et al.*, 1996; Wei *et al.*, 1996; Zehnder *et al.*, 2000). Among those that have evaluated PGPR-induced ISR to virus infection, only a few have addressed mechanistic issues. Maurhofer *et al.* (1998) showed that soil drenched with *P. fluorescens* CHA0 induced systemic protection against *Tobacco necrosis virus* (TNV) in tobacco. *Pseudomonas aeruginosa* 7NSK2 elicited ISR against *Tobacco mosaic virus* (TMV) (DeMeyer *et al.*, 1999). ISR elicited by both CHA0 and 7NSK2 required SA, as SA-deficient mutants failed to induce resistance against the respective viruses. Furthermore, enhanced ISR against TNV occurred when CHA0 was transformed with the SA biosynthesis gene (Maurhofer *et al.*, 1998). To date, however, no studies have shown that induced resistance by PGPR against a virus occurs upstream of SA or in a signaling pathway that is SA-independent.

Treatment of cucumber and tomato seed with *Serattia marcescens* strain 90-166 reduced disease severity of CMV and delayed the development of symptoms (Raupach *et al.*, 1996). In contrast, *P. fluorescens* WCS417r failed to induce resistance against TCV but did induce resistance against bacterial and fungal pathogens in *Arabidopsis* (Ton *et al.*, 2002). PGPR strains 90-166 and WCS417r were reported to protect plants against bacteria via a SA-independent pathway, as protection was observed on NahG plants (Pieterse *et al.*, 1998; Press *et al.*, 1997). The fact that both PGPR strains protect against bacteria but differ in their protection against viruses suggests the occurrence of a virus-specific signal pathway.

In this study, we show the occurrence of systemic protection against CMV in *A. thaliana* elicited by PGPR, and that the resistance follows a SA-independent signaling pathway.

Results

Effect of PGPR on systemic protection against CMV on Arabidopsis thaliana *Col-0 and NahG transgenic lines*

These experiments evaluated the response of Col-0 and NahG *Arabidopsis* plants treated with selected PGPR strains to inoculation with CMV. Evaluations included phenotypic responses, such as timing of appearance and severity of symptoms, and the amount of CMV accumulation in inoculated and non-inoculated leaves. CMV accumulation was determined by detection of viral coat protein using an anti-CMV coat protein antibody in the serological procedure enzyme-linked immunosorbent assay (ELISA). The two parameters, symptoms and CMV accumulation, are distinct aspects of the infection and disease processes and may or may not correlate with each other.

Plants inoculated with CMV in the control treatment developed systemic symptoms by 10 days post-inoculation (dpi). In those plants, symptoms initially included mild mosaic on the young, non-inoculated leaves with rapid progression to severe leaf deformation with stunting. In some cases, the plants had only a few surviving leaves by 21 dpi. When CMV disease severity was rated at 14 dpi, Col-0 plants treated with PGPR strains *Pseudomonas fluorescens* 89B61, *S. marcescens* 90-166, *Bacillus amyloliquefaciens* IN937a and *B. pumilus* SE34 as well as those in the BTH treatment had significantly lower disease severity ratings than those in the control treatment (Figure 1a). CMV disease severity was significantly lower for Col-0 plants treated with PGPR strain 90-166 than for plants treated with strains *P. fluorescens* 89B27, 89B61, IN937a, and *B. subtilis* IN937b. In addition, treatment with SE34 and BTH resulted in significantly lower disease severity ratings than treatment with strains 89B27 and IN937b.

The difference in disease severity of Col-0 plants treated with PGPR strain 90-166 relative to the control treatment is further illustrated in Figure 2. CMV infection of Col-0 plants at 14 dpi caused severe stunting with small, deformed leaves that were bunched together at the top of the plant (Figure 2a, plants 3 and 4). At 28 dpi, plants remained severely stunted with only limited extension of the flowering structures (Figure 2b, plant 1). In contrast, plants treated with PGPR strain 90-166 and infected with CMV developed a

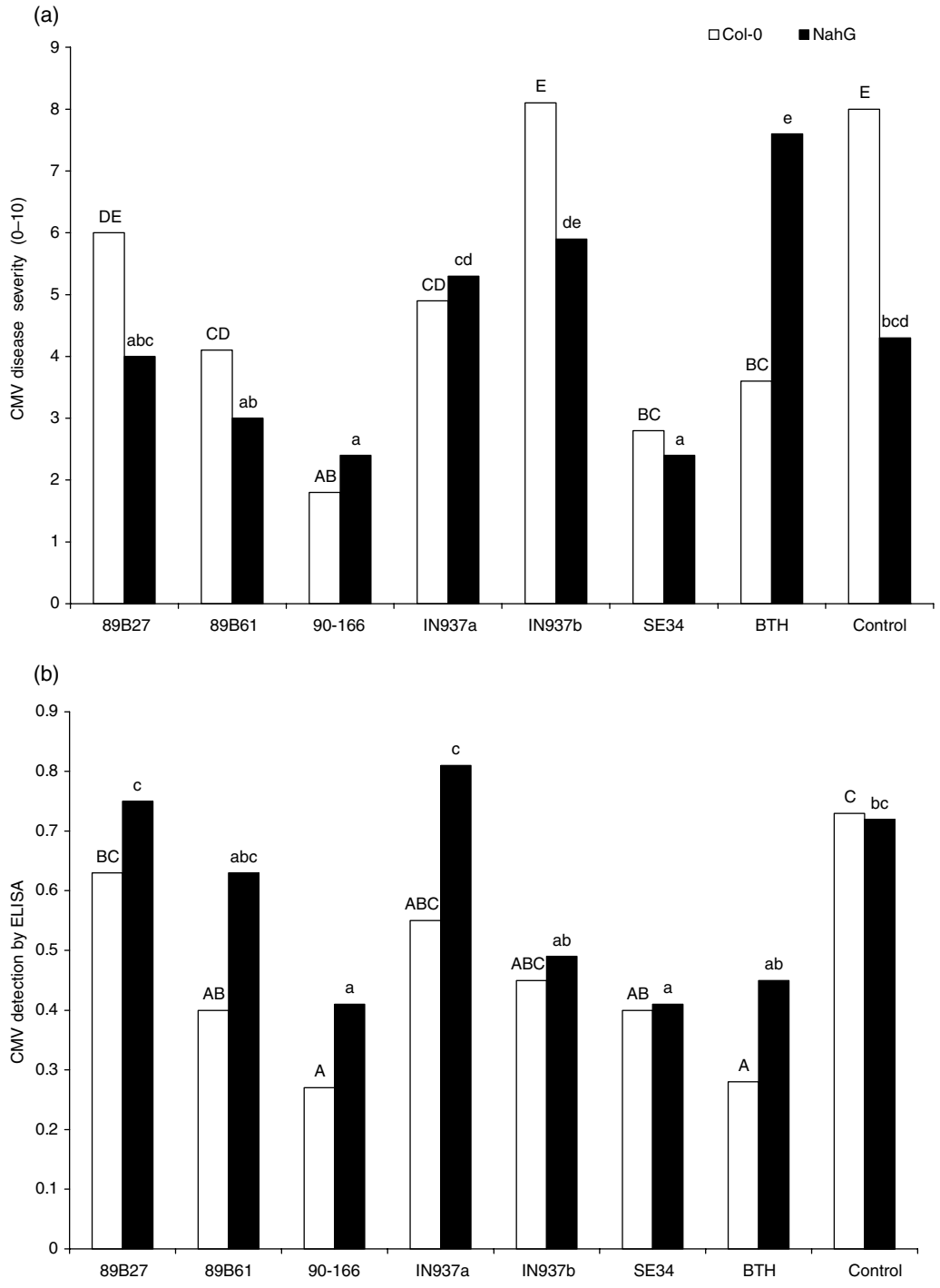


Figure 1. Response of *Arabidopsis thaliana* Col-0 and NahG plants treated with different PGPR strains or BTH and inoculated with *Cucumber mosaic virus* (CMV) at 14 days post-inoculation.

The PGPR strains are listed along the X-axis of each graph and are described in Experimental procedures.

(a) CMV disease severity among treatments using a 0–10 rating scale (described in Experimental procedures).

(b) CMV accumulation in non-inoculated leaves of *Arabidopsis* plants subjected to the different treatments as determined using enzyme-linked immunosorbent assay (ELISA). Disease severity and ELISA values represent the mean of 12 plants per treatment, arranged as a randomized complete block with each treatment within a block consisting of a row of four plants. Statistical comparisons in each graph were made among treatments within a single *Arabidopsis* line, for example, Figure 1(a), comparison of Col-0 plants subjected to the different treatments for disease severity. Different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

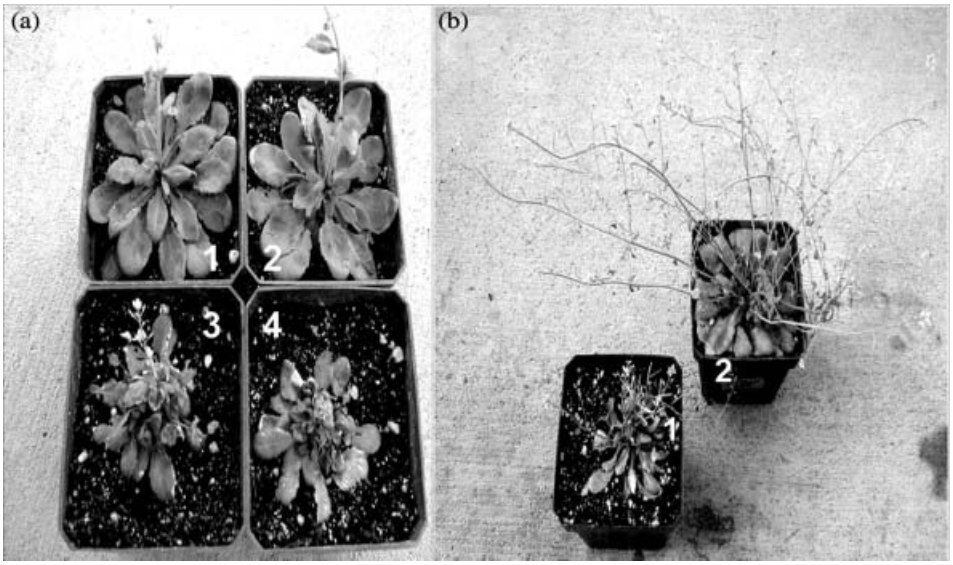


Figure 2. Disease severity of *Cucumber mosaic virus* (CMV)-infected *Arabidopsis thaliana* Col-0 plants treated with PGPR strain 90-166 relative to non-treated, control plants. (a) Plants evaluated at 14 days post-inoculation (dpi) with CMV. Plants 1 and 2 were treated with PGPR strain 90-166 and plants 3 and 4 represented non-treated, control plants. (b) Plants evaluated at 28 dpi with CMV. Plant 1 is a non-treated, control plant and plant 2 is a PGPR strain 90-166-treated plant.

mild chlorosis and mosaic on leaves but no apparent leaf distortion or stunting of the plant at 14 dpi (Figure 2a, plants 1 and 2). By 28 dpi, extensive inflorescence emergence and flowering were apparent for the 90-166 treated plant (Figure 2b, plant 2). While *Arabidopsis* plants are typically monocarpic, we frequently observed multiple inflorescence production with control and PGPR-treated plants that were not inoculated with CMV as well as with CMV-infected PGPR-treated plants.

In NahG plants, CMV disease severity was significantly greater for plants treated with BTH than for plants subjected to other treatments with the exception of PGPR strain IN937b (Figure 1a). In addition, NahG plants treated with PGPR strains 90-166 and SE34 had significantly lower disease severity ratings than plants in the control treatment and those plants treated with IN937a and IN937b.

CMV accumulation in non-inoculated (systemically infected) leaves was significantly less in Col-0 plants treated with PGPR strains 89B61, 90-166 and SE34 and the BTH treatment than in plants in the control treatment (Figure 1b). Col-0 plants treated with PGPR strain 90-166 and BTH had significantly lower amounts of CMV in non-inoculated leaves than plants treated with PGPR strain 89B27. In NahG plants inoculated with CMV, only PGPR treatments with 90-166 and SE34 resulted in significant reductions in CMV accumulation relative to the control and treatments with 89B27 and IN937a (Figure 1b).

Aboveground tissue fresh weight of Col-0 plants that were not challenged with CMV was greater for PGPR treatments with 89B27 and IN937a than for plants treated with PGPR strain IN937b and BTH (Table 1). Similarly, NahG plants that were not challenged with CMV and treated with PGPR strains 89B27 and IN937a resulted in significantly greater aboveground tissue fresh weight than plants treated with 90-166, IN937b, BTH and the non-treated control (Table 1). In addition, NahG plants treated with 89B61 weighed significantly more than plants treated with IN937b and BTH.

The effects on aboveground tissue fresh weight differed somewhat among treatments when inoculated with CMV (Table 1). Col-0 plants treated with PGPR strains 89B27, 90-166, IN937a, and SE34 had significantly greater aboveground tissue fresh weight than plants in the control treatment and those treated with IN937b. In addition, CMV-inoculated Col-0 plants treated with PGPR strains 89B27 and SE34 weighed significantly more than plants in treatments with 89B61, IN937b and BTH. For NahG plants inoculated with CMV, only plants treated with IN937a had significantly greater aboveground tissue fresh weight than plants in the control treatment. Growth of CMV-challenged NahG plants treated with IN937a was also greater than for plants in the IN937b and BTH treatments, while NahG plants treated with 89B61 and SE34 weighed significantly more than plants treated with IN937b or BTH. Interestingly, SE34 did not induce plant growth promotion in the non-virus treatment relative to the (non-PGPR) control treatment; however, CMV-infected Col-0 plants treated with SE34 were significantly larger than those in the control. This likely resulted from a protective effect of the SE34 treatment rather than enhanced growth as aboveground tissue weight of control plants was reduced by CMV infection.

Table 1 Growth of *Arabidopsis thaliana* plants treated with different PGPR strains or BTH with (virus) and without (no virus) inoculation with *Cucumber mosaic virus*

	Aboveground tissue fresh weight (g)[b]			
	Col-0		NahG	
Treatments[a]	No virus	Virus	No virus	Virus
89B27	5.30b[c]	4.44d	6.98c	4.30abc
89B61	3.55ab	3.11bc	5.76bc	5.21bc
90-166	3.85ab	3.80cd	3.55ab	4.10abc
IN937a	5.04b	3.61cd	6.90c	6.30c
IN937b	2.24a	1.69a	2.84a	2.30a
SE34	3.62ab	4.36d	4.90abc	5.90bc
BTH	2.80a	2.59abc	3.06a	2.21a
Control	3.34ab	2.06ab	4.16ab	3.81ab

[a]PGPR were applied to a soilless potting medium at the time the seed were sown and at 2 weeks post-germination of *A. thaliana* Col-0 and NahG plants. BTH was applied to the potting medium as a 0.33 mM solution.
[b]Numbers represent the mean fresh weight of aboveground tissues in grams measured at 21 days post-inoculation.
[c]Statistical comparisons are among treatments within a single column. The different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

Effect of PGPR strain 90-166 and its mutant strains on systemic protection against CMV on A. thaliana *Col-0 and its NahG transgenic lines*

PGPR strain 90-166, and the two mutant strains 90-166-1441 (deficient for SA production) and 90-166-2882 (deficient for siderophore production), significantly reduced CMV symptom severity in Col-0 and NahG plants compared with non-treated control plants (Table 2). Col-0 plants treated with strain 90-166 had significantly lower disease severity than plants treated with 90-166-2882, whereas no differences were observed among PGPR treatments in NahG plants. BTH treatment of Col-0 plants resulted in significantly lower CMV disease severity than was observed in the control, but no difference occurred in NahG plants.

CMV accumulation in the non-inoculated leaves of Col-0 and NahG plants subjected to the different treatments was evaluated by ELISA (Table 2). Strain 90-166 and its mutant, 90-166-1441, significantly reduced CMV accumulation in Col-0 plants relative to the non-treated control. In contrast, NahG plants treated with strain 90-166 accumulated significantly less CMV than plants treated with 90-166-1441, 90-166-2882 and for the non-treated control. CMV accumulation levels in the non-inoculated leaves of NahG plants did not differ among the 90-166 mutants and the non-treated control. Col-0 plants treated with BTH had significantly lower amounts of CMV in non-inoculated leaves when compared with all other treatments, whereas treatment of NahG plants with BTH reduced virus accumulation significantly relative to 90-166-2882 and the control.

The mean fresh weight of aboveground tissues of Col-0 plants was significantly greater for CMV-inoculated plants treated with PGPR strains 90-166, 90-166-1441 and 90-166-2882 than for plants in the BTH treatment and the non-treated control (Table 2). In contrast, no differences were observed among PGPR treatments and the non-treated control for NahG plants, although BTH-treated plants weighed significantly less than plants in the other treatments.

Table 2 Response of *Arabidopsis thaliana* ecotype Col-0 and transgenic line NahG plants treated with PGPR strain 90-166 or each of two mutants, 90-166-1441 (salicylic acid-negative mutant) and 90-166-2882 (siderophore-negative mutant) to inoculation with *Cucumber mosaic virus* (CMV)

	CMV disease severity[b]		CMV detection by ELISA (405 nm)[c]		Foliar fresh weight (g)[d]	
Treatments[a]	Col-0	NahG	Col-0	NahG	Col-0	NahG
90-166	3.8a[e]	1.3a	1.51b	0.70ab	1.82b	1.72b
90-166-1441 (SA–)	5.6ab	1.9a	1.51b	1.67cd	1.67b	1.67b
90-166-2882 (Sid–)	6.4b	2.6a	1.75bc	2.27d	1.97b	2.03b
BTH	6.1b	4.7bc	0.62a	1.19bc	0.94a	0.91a
Control	8.7c	6.4b	2.00c	1.99d	1.03a	1.67b

[a]PGPR were applied to a soilless potting medium at the time the seed were sown and at 2 weeks post-germination of *A. thaliana* Col-0 and NahG plants. BTH was applied to the potting medium as a 0.33 mM solution.
[b]Numbers represent the mean disease severity rating at 14 days post-inoculation (dpi) using a 0–10 scale as described in Experimental procedures. Each treatment consisted of 12 plants arranged in a randomized complete block design with four plants per treatment per block.
[c]CMV was detected by ELISA in non-inoculated leaves at 14 dpi. Samples were considered positive for CMV infection when the mean ELISA value was greater than the mean plus three standard deviations for comparable healthy control samples.
[d]Numbers represent the mean fresh weight of aboveground tissues in grams measured at 21 dpi.
[e]Statistical comparisons are among treatments within a single column. The different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

Time course *study with* PGPR *strain 90-166 on protection of A. thaliana Col-0, NahG,* npr1-1 *and* fad3-2 fad7-2 fad8 *lines against CMV*

CMV accumulated to a significantly lower level in the inoculated leaves of Col-0 plants treated with BTH than with PGPR strain 90-166 and the non-treated control (Table 3). When the non-inoculated leaves of Col-0 plants were tested for CMV accumulation, significantly lower amounts of virus were detected in plants treated with 90-166 and BTH than in the non-treated control at 7 dpi. In addition, significantly less CMV was detected in BTH-treated plants at 7 dpi than in plants treated with strain 90-166. No differences occurred among treatments for CMV accumulation in non-inoculated leaves at 14 and 21 dpi (Table 3).

Treatment of NahG plants with PGPR strain 90-166 or BTH did not reduce CMV accumulation in inoculated leaves at 7 dpi; however, significantly less virus accumulated in the non-inoculated leaves of 90-166-treated plants at 7 and 14 dpi than for plants treated with BTH and the non-treated control (Table 3). No difference in CMV accumulation occurred among treatments of NahG plants at 21 dpi.

In *npr1-1* plants, no differences in CMV accumulation occurred in inoculated leaves among treatments at 7 dpi (Table 3). In non-inoculated leaves, however, *npr1-1* plants treated with strain 90-166 had significantly lower amounts of CMV than plants in the BTH and control treatments at 7 dpi, and in the control treatment at 14 dpi.

No differences were observed for CMV accumulation in inoculated leaves at 7 dpi or non-inoculated leaves at 7 and 14 dpi among treatments in *fad3-2 fad7-2 fad8* plants (Table 3). Treatment of *fad3-2 fad7-2 fad8* plants with BTH, however, did result in significantly less CMV accumulation than in plants treated with PGPR strain 90-166 at 21 dpi.

At 21 dpi, plants were rated for CMV disease severity in the different *A. thaliana* lines (Figure 3). Treatment of Col-0, NahG and *npr1-1* plants with PGPR strain 90-166 resulted in significantly lower disease severity ratings compared with the non-treated control. BTH treatment also significantly reduced disease severity in Col-0 plants, but not in the other *Arabidopsis* lines. Neither PGPR strain 90-166 nor BTH treatments resulted in reduced CMV disease severity in *fad3-2 fad7-2 fad8* plants relative to the non-treated control.

Confirmation of jasmonic acid involvement by PGPR strain 90-166

Induction of JA signaling was tested using an *Arabidopsis* transgenic line containing the JA indicator gene *PDF1.2* fused to a GUS reporter gene (Brown *et al.*, 2003) at 1, 3, and 10 days after application. Untreated (control) plants showed no sign of GUS expression. Treatment of seedlings with methyl jasmonate (MeJA) or PGPR strain 90-166 induced

Table 3 *Cucumber mosaic virus* (CMV) accumulation in inoculated and non-inoculated leaves of *Arabidopsis thaliana* lines treated with either PGPR strain 90-166 or BTH

	Detection of CMV by ELISA (405 nm)[b]							
	Col-0		NahG		*npr1-1*		*fad3-2 fad7-2 fad8*	
Treatments[a]	Inoc	Nonin	Inoc	Nonin	Inoc	Nonin	Inoc	Nonin
7 dpi								
90-166	0.60b[c]	0.94b	0.64a	0.36a	0.81b	0.52a	0.90a	0.90a
BTH	0.56a	0.21a	0.85a	1.00b	0.92b	0.92b	0.84a	0.84a
Control	0.73b	1.24c	0.91a	0.73b	0.98b	0.98b	0.79a	0.97a
14 dpi								
90-166	nt	0.93a	nt	0.51a	nt	0.56a	nt	1.17a
BTH	nt	0.90a	nt	0.83b	nt	0.82ab	nt	1.05a
Control	nt	1.15a	nt	0.99b	nt	0.88b	nt	1.08a
21 dpi								
90-166	nt	0.63a	nt	0.61a	nt	0.81a	nt	1.04b
BTH	nt	0.54a	nt	0.64a	nt	0.66a	nt	0.83a
Control	nt	0.71a	nt	0.74a	nt	0.56a	nt	0.96ab

[a]PGPR were applied to a soilless potting medium at the time the seed were sown and at 2 weeks post-germination of *A. thaliana* Col-0, NahG, *npr1-1* and *fad3-2 fad7-2 fad8* plants. BTH was applied to the potting medium as a 0.33 mM solution. Each treatment consisted of 12 plants arranged in a randomized complete block design with four plants per treatment per block.

[b]CMV was detected by ELISA in inoculated leaves (inoc) at 7 days post-inoculation (dpi) and in non-inoculated leaves (nonin) at 7, 14, and 21 dpi. Samples were considered positive for CMV infection when the mean ELISA value was greater than the mean plus three standard deviations for comparable healthy control samples.

[c]Statistical comparisons are among treatments within a single column and within a single time period, for example, 7 dpi. The different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

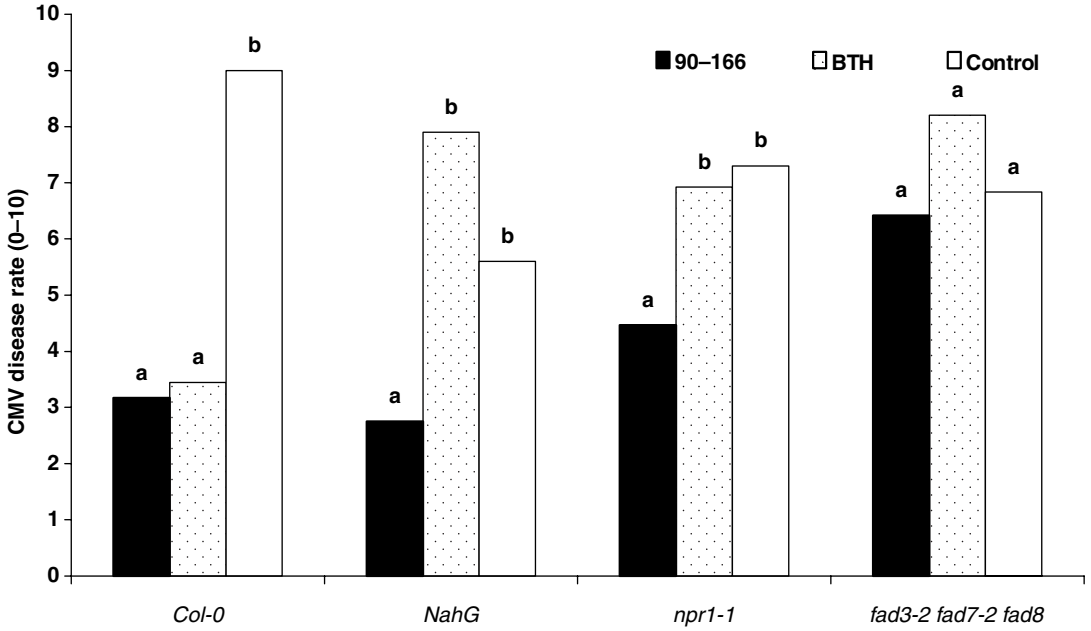
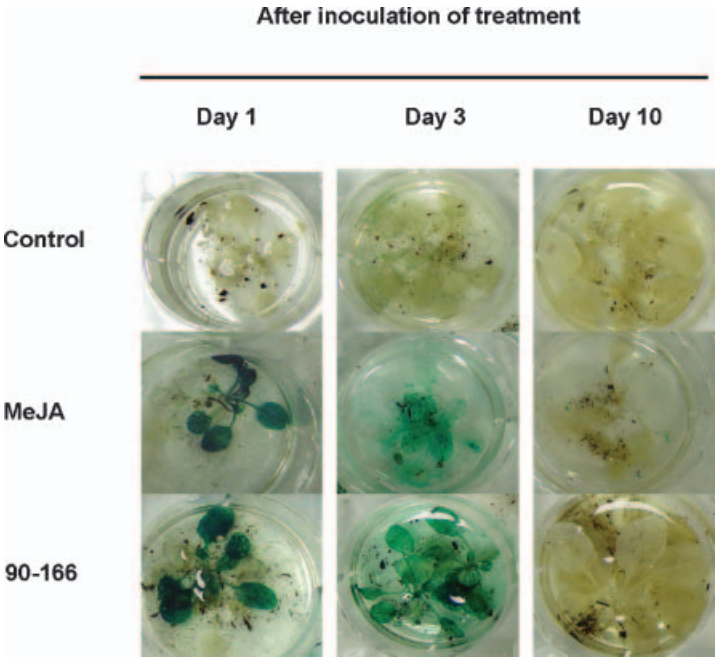


Figure 3. Disease severity of *Cucumber mosaic virus* (CMV)-inoculated *Arabidopsis thaliana* Col-0, NahG, *npr1-1* and *fad3-2 fad7-2 fad8* plants treated with PGPR strain 90-166 or BTH relative to non-treated, control plants at 21 days post-inoculation. CMV disease severity among treatments was determined using a 0–10 rating scale (described in Experimental procedures). Disease severity values represent the mean of 12 plants per treatment, arranged as a randomized complete block with each treatment within a block consisting of a row of four plants. Statistical comparisons were made among treatments (90-166, BTH and control) within an *Arabidopsis* genotype (e.g. Col-0). The different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

Figure 4. Time course and histochemical staining for *PDF1.2*::GUS expression after soil application of methyl jasmonic acid (MeJA) or PGPR strain 90-166. A 3 ml solution of 1 mM MeJA and 10^8 CFU of PGPR strain 90-166 were applied as a drench on *PDF1.2*::GUS plants grown in a soilless potting medium 1 week after transplanting. Histochemical GUS staining of whole plants treated with PGPR strain 90-166, MeJA and the control (1% ethanol) was performed at 1, 3 and 10 days after the respective treatments.



expression of *PDF1.2::GUS* at 1 and 3 days after each treatment (as illustrated by the blue/green stained plants in Figure 4) but not through 10 days after treatment. The 1% ethanol-treated plants (control) did not induce GUS gene expression. To determine the expression levels of *PR-1* and *PDF1.2* genes, RT-PCR experiments were performed with Col-0, *npr1-1*, and ethylene insensitive *etr1 Arabidopsis* plants 2 days after strain 90-166, BTH, and MeJA treatments

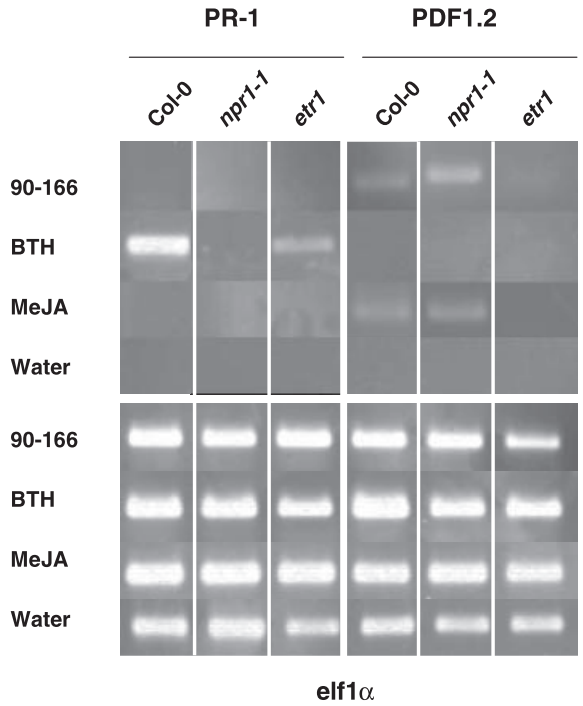


Figure 5. RT-PCR showing induction of *PR-1* and *PDF1.2* genes 2 days after inoculation of PGPR strain 90-166, salicylic acid, and jasmonic acid. Total RNA was extracted from the leaf tissue subjected to each of the treatments and first strand cDNA was synthesized using oligo d(T) primer. The cDNA was amplified by PCR using primers specific for *PR-1* or *PDF1.2* genes. PCR products were subjected to agarose gel electrophoresis and detected by staining with ethidium bromide. The amount of elongation factor-1 alpha (EF1α) transcripts was determined for every plant treatment combination and similar results were obtained. The names on the left side of the figure correspond to the different treatments: PGPR strain 90-166, BTH, MeJA and water.

(Figure 5). In the PDF1.2 signaling pathway, ethylene is involved in parallel with jasmonate (Penninckx *et al.*, 1998). We therefore used *etr1* mutant as a negative control for *PDF1.2* induction. We used *npr1-1* mutant as a negative control for PR-1 induction. Strain 90-166 did not induce *PR-1* transcript but did induce *PDF1.2* transcript in Col-0 and *npr1-1* plants. BTH-treated plants showed induction of *PR-1* in Col-0 and *etr1* plants but not *npr1-1* plants. No *PDF1.2* expression occurred in any of the *Arabidopsis* lines treated with BTH. MeJA did not induce *PR-1* expression in Col-0, *npr1-1* or *etr1* lines but did induce *PDF1.2* expression in Col-0 and *npr1-1* lines.

Discussion

PGPR strains that induced resistance against virus in cucumber and tomato in previous studies (Kloepper, 1996; Leeman *et al.*, 1996; Murphy *et al.*, 2000, 2003; Raupach *et al.*, 1996; Wei *et al.*, 1996; Zehnder *et al.*, 2000) also protected *A. thaliana* against infection by CMV. Of those PGPR strains tested in this study, *S. marcescens* 90-166 and *B. pumilus* SE34 significantly reduced symptom development and CMV accumulation on wild-type Col-0 and its NahG transgenic line. Ryals *et al.* (1996) showed that NahG plants, which do not accumulate SA as a result of the expression of the bacterial salicylate hydroxylase *nahG* gene, lost SAR against several pathogens including virus. Our results show that some PGPR strains protect NahG *Arabidopsis* plants against CMV suggesting a SA-independent signaling pathway.

Previous studies suggested that several bacterial determinants such as siderophores, SA, and lipopolysaccharides (LPS) contributed to ISR (Leeman *et al.*, 1995, 1996; Maurhofer *et al.*, 1998). Many rhizosphere bacteria produce SA as a siderophore under iron-deficient conditions and may activate a SA-dependent signaling pathway in ISR. However, SA produced by *S. marcescens* 90-166 was not a primary determinant of ISR in cucumber and tobacco against fungal and bacterial pathogens (Press *et al.*, 1997, 2001). The SA-negative mutant strain 90-166-1441 also induced resistance against *P. syringae* pv. tabaci in tobacco cv. Xanthi-nc and its NahG-transgenic line at similar levels as the parental strain (Press *et al.*, 1997).

For confirmation of SA-independent protection against CMV in the current study, the SA-negative mutant strain 90-166-1441 was tested in NahG *Arabidopsis* plants. Our results indicated that 90-166-1441 significantly reduced CMV symptom severity in NahG *Arabidopsis* (Table 2). The opposite was found with *P. aeruginosa* strain 7NSK2 and its SA-deficient mutant, which did not protect NahG plants against TMV in tobacco (DeMeyer *et al.*, 1999). Hence, ISR by 7NSK2 in tobacco is dependent on SA produced by the bacterium. Similarly, when the SA-biosynthetic gene cluster *pchDCBA* from *P. aeruginosa* PAO1 under the control of a constitutive promoter was introduced into non-SA-producing P3, it elicited significant protection against TNV compared to that of the control (Maurhofer *et al.*, 1998). Introduction of the gene *pchDCBA* into SA-producing CHA0 increased SA production but did not enhance ISR to TNV (Maurhofer *et al.*, 1998). Strain WCS417r produces SA *in vitro* under iron-limiting conditions and elicits ISR in wild-type and NahG transgenic *Arabidopsis* against *P. syringae* pv. tomato. However, strain WCS417 failed to elicit ISR against *Turnip crinkle virus* (TCV) in *Arabidopsis* (Ton *et al.*, 2002). Our results show that a PGPR strain can induce resistance to a virus by a SA-independent pathway because strain 90-166 and its SA-deficient mutant 90-166-1441 reduced symptom development in both Col-0 and NahG plants (Table 2). The lack of *PR-1* gene induction by strain 90-166 provides further evidence of SA-independence because the *PR-1* gene is commonly used as an indicator of SA signaling (Figure 5). This result corresponds to that of strain WCS417, which elicited ISR without expression of the *PR-1* gene (Pieterse *et al.*, 1998). Interestingly, strain WCS417

protected *Arabidopsis* against bacterial pathogens but not against virus through a SA-independent signaling pathway (Ton *et al.*, 2002).

In addition to SA, siderophores have also been associated with ISR, especially under iron-limited conditions (Press *et al.*, 1997, 2001). Plants grown on medium amended with the synthetic iron chelator EDDHA demonstrated enhanced levels of ISR (Leeman *et al.*, 1996; Press *et al.*, 2001). Our findings demonstrate that the siderophore-mutant PGPR strain 90-166-2882 significantly reduced CMV symptoms relative to the non-treated control but less than the wild-type strain 90-166 in Col-0 plants. Previous results showed that strain 90-166-2882 was unable to induce ISR against *C. orbicularie* on cucumber and *P. syringae* pv. tabaci on tobacco (Press *et al.*, 1997). These results suggest that PGPR strain 90-166 activates different signal pathways against bacterial and viral pathogens.

In this study, we demonstrated that treatment with PGPR strain 90-166 resulted in systemic protection against CMV in *npr1-1* but not in *fad3-2 fad7-2 fad8* plants based on CMV accumulation at 7 and 14 dpi. Wong *et al.* (2002) recently reported that SA and non-lethal concentrations of cyanide and AA could induce resistance to TVCV in *npr1-1*. These studies suggest that induced resistance against virus probably does not require the *NPR1-1* gene. In addition, we showed that treatment with strain 90-166 did not result in reduced symptom development and CMV accumulation levels in *fad3-2 fad7-2 fad8* plants, indicating that protection induced against CMV by strain 90-166 was through a JA-dependent pathway. As illustrated in Figure 4, JA signaling is induced in *Arabidopsis* seedlings treated with PGPR strain 90-166. PDF1.2, an indicator of JA signaling, was induced by strain 90-166 confirming the involvement of JA signaling (Figure 5). Similarly, strain 90-166 elicited ISR against two bacterial pathogens through JA-dependent signaling (Ryu *et al.*, 2003). In contrast to strain WCS417, the PGPR strain 90-166 did protect *Arabidopsis* plants against CMV by the same SA-independent and JA-dependent signaling pathway. This may be explained by the existence of a novel ISR signaling pathway against virus or a branch in the pathway downstream of JA signaling to elicit ISR with one directed against bacteria and the other against virus.

We sometimes observed a disparity between disease severity and CMV accumulation levels in non-inoculated leaves. At 21 dpi, plants treated with PGPR strain 90-166 had significantly lower disease severity ratings in each of the *Arabidopsis* lines except *fad3-2 fad7-2 fad8*, while treatment with BTH reduced CMV disease severity only in wild-type Col-0 plants (Figure 3). The likely reason for these results is that BTH induces SAR through SA signaling, which was abolished in *npr1-1* plants. In contrast to the reduction in disease severity (Figure 1), treatment with strain 90-166 did not result in reduced amounts of CMV accumulation in the non-inoculated leaves of *npr1-1* plants compared to the control at 21 dpi (Table 3). In NahG plants, significantly lower amounts of CMV were detected in non-inoculated leaves at 7 and 14 dpi but not at 21 dpi (Table 3), and yet, disease symptoms remained significantly less severe at 21 dpi (Figures 1 and 3). The treatment of *Arabidopsis* plants with PGPR strain 90-166 had no apparent effect on the amounts of CMV accumulation in inoculated leaves but treated plants had reduced amounts of virus in non-inoculated leaves at 7 and 14 dpi, relative to the non-treated control. While the amount of CMV in non-inoculated leaves at 21 dpi did not differ among treatments, disease severity was significantly less in Col-0, NahG and *npr1-1* plants. These results suggest that the resistance against CMV induced by treatment with strain 90-166 affected systemic infection in the form of a delay in movement to young tissues and/or a delay in accumulation in young tissues thereby affecting development of symptoms.

In conclusion, our data show that protection of *Arabidopsis* against CMV by a plant growth-promoting rhizobacterium follows a pathway that is independent of SA and NPR1 but dependent on jasmonic acid. Further confirmation was provided by the expression of *PR-1* and *PDF1.2* genes and that PGPR strain 90-166 induces the *PDF1.2* gene as a JA signaling indicator. These results indicate that strain 90-166 elicits ISR against CMV via a signaling pathway not previously identified for virus resistance.

Experimental procedures

PGPR strains and inocula preparation

The six wild-type PGPR strains used were *S. marcescens* 90-166, *B. pumilus* SE34, *P. fluorescens* 89B61, *P. fluorescens* 89B27, *B. amyloliquefaciens* IN937a, and *B. subtilis* IN937b. These strains were previously shown to induce systemic protection in cucumber and tomato against viral diseases (Murphy *et al.*, 2000; Raupach *et al.*, 1996; Zehnder *et al.*, 1999). Prior to use, each PGPR strain was stored at −80°C in tryptic soy broth (TSB) amended with 20% glycerol. The strains were removed from ultra-cold storage, streaked onto tryptic soy agar (TSA), and incubated at 28°C for 24 h to check for purity. After transferring single colonies, bacteria were incubated for 2 days at 27°C on TSA, and were then scraped off plates into sterilized distilled water (SDW). The resulting bacterial suspensions were adjusted with SDW to 10^9 CFU ml^{-1} using a spectrophotometer ($OD_{600\ nm}$).

Arabidopsis lines and preparation

The *A. thaliana* SA hydroxylase gene transgenic NahG line and the *npr1-1* line (which does not express PR proteins) were obtained from Dr Xinnion Dong, Duke University, Durham, NC, USA. The JA-insensitive mutant line *fad3-2 fad7-2 fad8* (Vijayan *et al.*, 1998) was provided by Dr John Browse, Washington State University, Pullman, WA. *PDF1.2* promoter fusion GUS *Arabidopsis* was provided by Dr Willem F. Broekaert, Katholieke Universiteit Leuven, Heverlee-Leuven, Belgium.

All mutant and transgenic lines listed above were derived from parental *A. thaliana* Hynn. ecotype Columbia (Col-0) and its ethylene insensitive mutant *etr1*, which were obtained from the Ohio State University Stock Center (Columbus, OH, USA). Seeds were surface-sterilized with 70% ethanol for 1–2 min followed by treatment with 1% sodium hypochlorite for 20 min prior to planting in a peat-based soilless potting medium (Speedling Inc., Bushnell, FL, USA) at 23 ± 3°C under natural light in a greenhouse. Two-week-old seedlings were transferred to 10 cm square pots with one plant per pot. Preparation of the PGPR treatment involved mixing 1 l of each bacterial suspension into 10 l of soilless potting medium. The final concentration of each PGPR strain was 10^8 CFU g^{-1} soil. Stock solutions of BTH (Syngenta, Research Triangle Park, NC, USA) at 0.33 mM were freshly prepared in SDW for each experiment and applied as a soil-drench. A non-treated control treatment consisted of an equivalent volume of water.

Cucumber mosaic virus *inoculations*

CMV strain Fny was originally obtained from Dr Peter Palukaitis (Scottish Crop Research Institute, Invergowrie, UK) and was used throughout this project. CMV was maintained in *Nicotiana tabacum* cultivar Kentucky 14 by mechanical passage in a temperature-controlled greenhouse. The CMV inoculum used throughout these experiments consisted of systemically infected Kentucky 14 tissue ground in 50 mM potassium phosphate buffer, pH 7.0, and 10 mM sodium sulfite (1 g tissue: 10 ml buffer). All inoculation materials were chilled at 4°C prior to inoculation and maintained on ice during inoculation. Plants were inoculated at 4 weeks after planting. A fresh preparation of CMV inoculum was used for each of five rows of plants (see experimental designs outlined for each experiment). CMV was mechanically inoculated by rub inoculation onto the oldest three leaves (each of which was lightly dusted with carborundum prior to inoculation) of each *Arabidopsis* plant at 7 days after the PGPR or BTH treatments. Plants that represented 'mock' inoculation were mechanically (rub) inoculated using 50 mM potassium phosphate, pH 7.0, containing 10 mM sodium sulfite in the same manner as plants inoculated with CMV inoculum.

At 14 days after inoculation, disease severity was measured using a 10-point rating scale: 0 = no symptoms; 2 = mild deformation and mosaic of the youngest two leaves; 4 = pronounced leaf deformation and mosaic of the youngest two leaves with progression of symptoms into sequentially older leaves; 6 = pronounced leaf deformation and mosaic progressed beyond the two youngest leaves with all leaves expressing some form of CMV-induced symptoms; 8 = similar symptoms as described for a rating of 6 with plants also being stunted in growth (note that this stunting included both reduced internode extension and smaller leaves); 10 = plants were severely stunted with a majority of leaves being small, severely deformed and tightly bunched together.

The fresh weight of aboveground tissues of individual plants was determined at 14 days after CMV inoculation.

Enzyme-linked immunosorbent assay (ELISA)

Detection of CMV in *Arabidopsis* tissue was determined by antigen-coated plate, indirect ELISA using an anti-CMV coat protein antibody as described by Garcia-Ruiz and Murphy (2001). Inoculated leaves were tested for CMV infection at 7 dpi and non-inoculated leaves were tested at 7, 14 and 21 dpi. At each period of testing for CMV accumulation in leaf tissues, samples were collected from a distinct set of plants that had not been sampled for previous tests. Leaf samples of 50–100 mg were ground in 1 ml of 50 mM carbonate buffer, pH 9.6, in a 1.5 ml Eppendorf tube using a plastic homogenizer. Samples were considered positive for the presence of CMV when the absorbance value was greater than the mean plus three standard deviations of the healthy control samples. Each ELISA test included a series of known concentrations of purified CMV to standardize the ELISA reactions among tests. ELISA reactions were recorded with a Sunrise microtiter plate reader (Phenix Research Products, Hayward, CA, USA).

Response of A. thaliana *ecotype Col-0 and SA-insensitive transgenic line NahG to inoculation by CMV*

The *A. thaliana* ecotype Col-0 and the SA-insensitive transgenic line NahG were treated with each of the six PGPR strains, BTH or water (used as a control) and evaluated for their response to CMV inoculation. The experiments were conducted under greenhouse conditions with each of the PGPR treatments amended into soilless potting medium as described above. Two milliliters of BTH (0.33 mM solution) was applied directly to each plant as a drench to the soilless potting medium immediately after being transplanted to 10 cm^2 pots (one plant per pot). Plants were then rub-inoculated with CMV one week later as described above. At 14 dpi, plants were evaluated for disease severity using the 0–10 rating scale and leaf tissues were tested for CMV infection by ELISA. This experiment was designed as a randomized complete block with each treatment within a block consisting of a row of four plants. The treatments were randomly arranged among blocks with three replications. This experiment was performed three times.

Effect of PGPR strain 90-166 and related mutant strains on systemic protection against CMV and plant growth of A. thaliana *ecotype Col-0 and transgenic line NahG*

PGPR strain 90-166 and two related mutants were evaluated: strain 90-166-1441 does not produce SA and strain 90-166-2882 is unable to induce systemic resistance in cucumber and tobacco (Press *et al.*, 1997, 2001). Strain 90-166-2888 has a mutation in the *entA* gene, which encodes an enzyme of the cathechol siderophore biosynthetic pathway (Press *et al.*, 2001). The bacterial growth conditions and application to the soil were the same as described in the previous experiment. Control treatments included BTH and water as described above. Disease severity ratings were based on the 0–10 scale and CMV accumulation in non-inoculated leaves was determined by ELISA at 14 dpi as described above. This experiment was designed as a randomized complete block with each treatment consisting of a row of four plants. The treatments were randomly arranged among blocks with three replications. The experiment was performed three times.

Time course study with PGPR strain 90-166 on protection of A. thaliana *Col-0, NahG transgenic,* npr1-1 *and* fad3-2 fad7-2 fad8 *lines against CMV*

A time course study was conducted to evaluate CMV infection in *A. thaliana* ecotype Col-0 and transgenic lines NahG, *npr1-1* and *fad3-2 fad7-2 fad8* after treatment with PGPR strain 90-166. The PGPR strain 90-166, BTH and water treatments were as described above. CMV disease severity was measured using the 0–10 scale at 21 dpi. CMV accumulation was determined by ELISA in inoculated leaves at 7 dpi and in non-inoculated leaves at 7, 14 and 21 dpi. This

experiment was designed as a randomized complete block with each treatment consisting of a row of four plants. The treatments were randomly arranged among blocks with three replications. The experiment was performed three times.

GUS staining

Histochemical GUS staining was performed as described previously (Brown *et al.*, 2003). The three plants treated with strain 90-166 and methyl jasmonic acid were collected for GUS staining at 1, 3 and 10 days after drenching 1 mM MeJA in control (1% ethanol) or PGPR strain 90-166 in SDW. Whole plants were immersed in a staining solution (2 mM X-Gluc in *N,N*-dimethylformamide, 100 mM NaH_2PO_4, 10 mM Na_2EDTA, 0.5 mM K ferrocyanide, and 0.1% Triton X-100, pH 7.0) followed by incubation at 37°C overnight in the dark. The plants were cleared of chlorophyll by treatment with 50% ethanol after staining at room temperature for 24 h. Stained samples were observed and photographed with a digital camera (Sony, Park Ridge, NJ, USA).

RNA extraction and RT-PCR analysis

Total RNA from leaf tissue was isolated from *Arabidopsis* plants treated with strain 90-166, 300 μM BTH, 100 μM JA, or water 2 days after treatment using TRIzol® reagent (Invitrogen Co., Carlsbad, CA, USA) according to manufacturer's recommendations and treated with RNase-free DNase. A reverse transcriptase (RT) reaction was performed on 1–5 μg of total RNA with 200 units of RNase H-RT™ (Invitrogen Co.), 500 ng oligo $d(T)_{12–16mer}$ primer and 500 μM dNTPs in a final volume of 20 μl. Semi-quantitative RT-PCR was performed in a final volume of 75 μl using 1.5 μl of cDNA, 1X PCR buffer (with 1.5 mM $MgCl_2$), 200 μM dNTP, 200 nM of each gene specific primers and 1.5 units of *Go-Taq* polymerase (Promega Co., Madison, WI, USA). To ensure that only host genes and not the viral RNA transcripts are amplified, the RT reactions were performed using oligo d(T) primers. As a loading control, parallel reactions using elongation factor primers were carried out. The details of all the primers employed for the RT-PCR reactions are *AtPR-1* forward: ATG AAT TTT ACT GGC TAT TCT CG and *AtPR-1* reverse: CAT TAG TAT GGC TTC TCG TTC, *AtPDF1.2* forward: ATG GCT AAG TTT GCT TCC AT and *AtPDF1.2* reverse: TTA ACA TGG GAC GTA ACA GA, and *AtEIF-4A2* forward: CAAGAGAATCTTCTTAGGGGTATCTATGC and *AtEIF-4A2* reverse: GGTGGGAGAAGCTGGAATATGTCATAG. Primers were designed from *Arabidopsis* PR-1 gene (GenBank accession no. M90508). PCR conditions used for all the genes were used for the first cycle of 4 min at 94°C, 30 sec at 55°C and 30 sec at 72°C was followed by 30 sec at 92°C, 30 sec at 59°C and 30 sec at 72°C for 34 more cycles. A 5 μl aliquot was removed from each reaction after 25, 30, and 35 cycles. The aliquots were analyzed on a 1% agarose gel stained with ethidium bromide. Expected product size was confirmed by a 1 kbp DNA ladder (Life Technologies, Rockville, MD, USA). Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a Ultra-Lum CCD camera (Claremont, CA, USA).

Data analysis

Data were subjected to analysis of variance using JMP software (SAS Institute Inc., Cary, NC, USA). When a significant *F* test was obtained at $P = 0.05$, separation of treatment means was accomplished by Fisher's protected LSD.

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