

Induction of systemic resistance against *Cucumber mosaic virus* by *Penicillium simplicissimum* GP17-2 in *Arabidopsis* and tobacco

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The plant growth-promoting fungus, *Penicillium simplicissimum* GP17-2, was evaluated for its ability to induce resistance against *Cucumber mosaic virus* (CMV) in *Arabidopsis thaliana* and tobacco plants. Treatment with barley grain inoculum (BGI) of GP17-2 significantly enhanced fresh weight, dry weight and leaf number of *A. thaliana* and tobacco plants 6 weeks after planting. Two weeks after CMV inoculation, all plants treated with BGI of GP17-2 or its culture filtrate (CF) showed a significant reduction in disease severity compared with non-treated control plants, which exhibited severe mosaic symptoms by the end of the experiment. The enzyme-linked immunosorbent assay (ELISA) demonstrated that CMV accumulation was significantly reduced in plants treated with GP17-2 or its CF relative to control plants. Based on RT-PCR, plants treated with GP17-2 (BGI or CF) also exhibited increased expression of regulatory and defence genes involved in the SA and JA/ET signalling pathways. These results suggested that multiple defence pathways in *A. thaliana* and tobacco were involved in GP17-2-mediated resistance to CMV, although neither the transgenic *NahG* line, nor the *npr1*, *jar1* or *ein3* mutants disrupted the response in *A. thaliana*. This is the first report to demonstrate the induction of systemic resistance against CMV by GP17-2 or its CF.

Keywords: *Arabidopsis thaliana*, *Cucumber mosaic virus*, induced systemic resistance, *Nicotiana tabacum*, *Penicillium simplicissimum* GP17-2, plant growth-promoting fungi

Introduction

Cucumber mosaic virus (CMV) is one of the most destructive plant viruses, affecting the production of many crops in the world (Roossinck, 1999). It is difficult to control CMV because of its broad host range – it attacks more than 800 plant species – and its transmission in a non-persistent manner by more than 60 species of aphids (Palukaitis *et al.*, 1992). Many studies have been published on the development of biological control agents, such as plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) (Lyon & Newton, 1997).

Plants utilize different defence mechanisms against pathogen attack. Some of these mechanisms can be triggered by certain stimuli prior to an attack, resulting in a reduction in disease severity (Ryu *et al.*, 2004; Lee *et al.*, 2005; Kang *et al.*, 2007). The induction of systemic resistance has potential as an alternative method of crop pro-

tection. Yarwood (1960) first described the plant response to viral infection as a localized acquired resistance to *Tobacco mosaic virus* (TMV) in bean leaves. Ross (1961) showed that the infection of lower leaves of tobacco by TMV induced resistance to subsequent TMV infections in distal uninfected leaves, referred to as ‘systemic acquired resistance’ (SAR). SAR can be triggered by exposing the plant to pathogenic microbes or artificially with chemicals, such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA) or benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Métraux *et al.*, 1991; Görlach *et al.*, 1996; Vallad & Goodman, 2004). Systemically induced resistance is a promising strategy to control plant diseases, as it affects numerous pathogens (Doornbos *et al.*, 2011). Induced systemic resistance (ISR) is defined as a phenomenon by which a plant exhibits an increased level of resistance to pathogen infection after the appropriate stimulation by avirulent or non-pathogenic microbes.

PGPR and PGPF are classes of soilborne microbes with beneficial effects on plant growth and the induction of defence resistance (Hossain *et al.*, 2007; Ryu *et al.*, 2007; Sultana *et al.*, 2009). Several studies have established the role of selected strains of PGPR in ISR against CMV (Ipper *et al.*, 2008; Wang *et al.*, 2009).

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One example of a PGPF is *Penicillium simplicissimum* GP17-2, which was isolated from the rhizosphere of zoysiagrass (*Zoysia tenuifolia*) and enhanced the growth of different agricultural crops (Hyakumachi, 1994; Shivanna *et al.*, 1996a; Hossain *et al.*, 2007). The barley grain inoculum (BGI) and culture filtrate (CF) of GP17-2 were shown to induce ISR responses in cucumber (Shivanna *et al.*, 1996b; Koike *et al.*, 2001) and *A. thaliana* plants (Hossain *et al.*, 2007).

In spite of major advances in the understanding of plant defence responses, little information is available concerning PGPF-mediated induced resistance against viruses. Correlated with the onset of ISR is the enhanced production of the plant antioxidant protective enzyme, peroxidase (Shoman *et al.*, 2003), and the activation of certain plant defence genes that produce pathogenesis-related (PR) proteins, the exact modes of action of which are not entirely understood (Shehata & El-Borollosy, 2008).

Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are recognized as key players in the regulation of the signalling pathways involved in SAR and ISR (van Loon *et al.*, 2006; von Dahl & Baldwin, 2007). Both SAR and ISR pathways require *NPR1* function (Dong, 1998; Pieterse *et al.*, 1998). Moreover, the *Co1* gene is a key regulatory component of the JA signalling pathway and is required for plant fertility and defence responses (Xie *et al.*, 1998). While SA accumulation is required for SAR (Sticher *et al.*, 1997), ISR is independent of SA, but relies on JA- and ET-mediated pathways (Yan *et al.*, 2002). Some of the PGPR strains that trigger ISR are linked to the induction of SAR (Ryu *et al.*, 2004). In *A. thaliana*, a microarray analysis of plants that had been exposed to a variety of defence-inducing treatments revealed that more than 50 defence-related genes are co-induced by SA and JA, suggesting that the two signals coordinately regulate these genes (Schenk *et al.*, 2000). Martinez *et al.* (2001) reported that SA, together with ET, coordinates the activation of defence mechanisms via an interaction between the two signalling pathways. Separate signalling pathways might have evolved to allow plants to fine-tune their defence responses, such that the appropriate defence combination against specific pathogens is deployed according to their virulence strategies (Kunkel & Brooks, 2002). Thuerig *et al.* (2006) reported *Penicillium chrysogenum*-induced resistance in *A. thaliana* that functioned independently of the SA and JA/ET signalling pathways. Sultana *et al.* (2009) showed that SA and JA/ET signalling pathways were modulated in *A. thaliana* by *Phoma* sp. GS8-3. Ryu *et al.* (2004) showed that PGPR-mediated protection against CMV in *A. thaliana* was independent of SA and *NPR1* but dependent on JA. Tobacco plants treated with a *Bacillus* sp. had enhanced expression of the PR genes *NPR1* and *Co1*, which was associated with an increased resistance to CMV (Wang *et al.*, 2009). Although many studies have reported the role of PGPR in the induction of ISR against CMV, little is known about using PGPF against CMV. Thus, the objective of this study was to evaluate the ability of the PGPF *P. simplicissimum*

GP17-2 and its CF to induce resistance against CMV in *A. thaliana* and tobacco plants.

Materials and methods

Plants and pathogens

Seeds of *A. thaliana* ecotype Columbia (Col-0) were provided by K. S. Park (NIAST, Suwon, Korea). The mutants *ein3* (Chao *et al.*, 1997), *jar1* (Staswick *et al.*, 1992) and *npr1* (Cao *et al.*, 1994) were obtained from the Nottingham Arabidopsis Stock Centre. The transgenic line *NabG* (Lawton *et al.*, 1995) was a personal gift. All mutants and transgenic *A. thaliana* plants were generated in a Col-0 background. Seeds of *Nicotiana tabacum* cv. Xanthi-nc and *Nicotiana benthamiana* were obtained from the Laboratory of Plant Pathology, Tohoku University, Japan, as was the yellow strain of *Cucumber mosaic virus* (CMV-Y). The virus was maintained in tobacco plants (cv. Xanthi-nc). The PGPF isolate *P. simplicissimum* GP17-2 was obtained from the Laboratory of Plant Pathology, Gifu University.

Barley grain inoculum (BGI)

Autoclaved barley grains (100 g in 100 mL water) were inoculated in a 500-mL Erlenmeyer flask with 10–15 mycelial discs (5 mm) transferred from the actively growing margin of 7-day-old potato dextrose agar (PDA; 2% agar) cultures of GP17-2. After 10–12 days of incubation at 25°C in the dark, the completely colonized barley grains were air-dried at room temperature (23–25°C). The dried BGI was ground to a 1- to 2-mm particle size and stored at 4°C until further use.

Cell-free culture filtrate (CF)

GP17-2 was cultured on PDA medium for 7 days. Twenty mycelial disks (5 mm) of GP17-2 culture were taken from the growing margin of a colony and transferred to a 500-mL Erlenmeyer flask containing 200 mL potato dextrose broth (PDB). The fungal culture was then maintained at room temperature (25°C) for 10 days without shaking. The crude culture filtrate was separated from the mycelia and filtered through two layers of Whatman No. 2 filter paper, and then filter-sterilized through a 0.22- μ m Millipore filter.

Plant growth conditions

For the barley grain inoculum experiments, sterilized paper pots (size 3.8 × 5 cm for *A. thaliana* and 5 × 7.5 cm for tobacco) were filled with approximately 40 g (*A. thaliana*) or 70 g (tobacco) autoclaved commercial potting medium 'Star-bed' (soilless, peat-based potting medium, containing humus, rock phosphate and composted plant materials, Kyodohiryo Co. Ltd., Aichi, Japan), and sown with *A. thaliana*, *N. tabacum* or *N. benthamiana* seeds (five seeds per pot). After

germination, plants were thinned to one plant per pot and maintained in a growth chamber under a 9-/15-h day/night cycle at 22°C for *A. thaliana* and a 12-/12-h day/night cycle at 25°C for tobacco.

For the culture filtrate experiments, *A. thaliana* and tobacco seeds were surface-sterilized by immersion in 70% ethanol for 2 min followed by 2% (v/v) NaOCl for 2 min, thoroughly rinsed three times in sterile distilled water, and vernalized for 2 days at 4°C in the absence of light. The seeds were soaked in 0.5 mL distilled water in Eppendorf tubes and stored in a refrigerator for 3 days at 4°C to synchronize germination. Using sterilized rockwool, five seeds were individually sown in each cube of rockwool, and thinned to one plant per cube after germination. The plants were irrigated with a 10-fold dilution of MGRL nutrient solution (Fujiwara *et al.*, 1992) once a week and maintained in the growth chambers as described previously.

Fungal and chemical pre-treatment

Autoclaved potting medium in sterile pots was amended with the powdered BGI (0.5% w/w) of GP17-2 and sown with *A. thaliana* and tobacco seeds. Autoclaved potting medium supplemented with an equal volume of autoclaved barley grain served as a control. In the case of the rockwool-grown plants, 4-week-old *A. thaliana* and tobacco plants were treated with 50% diluted CF of GP17-2 for 1 h, then the excess CF was washed away with distilled water. The control plants were similarly treated with 50% diluted PDB.

The synthetic SAR inducer benzothiadiazole (BTH), a functional analogue of SA (Lawton *et al.*, 1996), was included in these experiments as a positive control. The *A. thaliana* and tobacco plants grown in soil were treated with a soil drench of 0.3 mM BTH (Novartis Agro) 1 day prior to the challenge inoculation. However, in the case of the rockwool system, 4-week old *A. thaliana* and tobacco plants were treated for 1 h with a 5 mM BTH solution a day prior to the virus challenge inoculation and subsequently washed with distilled water.

Root colonization

Root colonization by GP17-2 was evaluated in *A. thaliana* and tobacco plants 7 weeks after planting for both GP17-2 and control treatments. Roots were collected from nine randomly selected plants, washed with tap water to remove the adhering soil, rinsed three times with sterile-distilled water, and blotted to dryness. They were then cut into 1-cm segments, plated onto PDA amended with 200 mg chloramphenicol L⁻¹ and incubated for 3–4 days at 25°C. After incubation, the GP17-2 colonies were identified by the colour and growth pattern of the mycelia and pigments produced. The isolation frequency of the fungus was determined by counting the number of colony-forming root segments among the 100 root segments plated per replicate, as described by Meera *et al.* (1995).

Cucumber mosaic virus inoculations

The CMV inoculum used throughout the experiments consisted of infected tobacco leaf tissue ground in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.002 M EDTA (1 g tissue per 50 mL sodium phosphate buffer). All inoculation materials were chilled at 4°C prior to the inoculation and maintained on ice during the inoculation. The plants were inoculated 4 weeks after planting by rubbing the inoculum onto the oldest leaf. Disease severity ratings for *A. thaliana* plants were: 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 (including both reduced internode extension and smaller leaves); and 10 = plants severely stunted with a majority of leaves being small, severely deformed and tightly bunched together (Ryu *et al.*, 2004). Disease severity was rated in tobacco plants by counting leaves with symptoms. The disease severity results were expressed as mean values of 10 samples in each treatment.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA, as described previously (Zehnder *et al.*, 2000) with some modifications, was used to determine CMV-Y concentration. Leaf samples were collected at 7 and 14 days post-inoculation (dpi), ground in 50 mM carbonate buffer (pH 9.6) and added to microtitre plates at a final dilution of 1:10 (g tissue mL⁻¹ buffer). The plates were incubated overnight at 4°C and then washed three times with phosphate-buffered saline containing Tween (PBS-T). Anti-CMV (primary antibody) was added to the plates at a concentration of 1 fg mL⁻¹ in PBS-T. The plates were incubated for 1.5 h at 37°C and washed three times with PBS-T. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase was diluted 1:7500 in PBS-T and added to the plates. The plates were incubated at 37°C for 1 h then washed three times with PBS-T, the substrate (*p*-nitrophenylphosphate at 1 mg mL⁻¹ in 10% diethanolamine, pH 9.8) was added and the reaction allowed to develop at room temperature. Absorbance values were read at 405 nm on a Bio-Rad model 550-microplate reader. ELISA was repeated in triplicate with four replicates per treatment and two leaves for each replicate.

Evaluation of plant growth

Shoot fresh weight and dry weight and number of leaves from the BGI-treated and non-treated *A. thaliana* and tobacco plants were recorded 6 weeks after planting. The experiment was repeated in triplicate with 10 plants per replicate.

RT-PCR analysis

For RNA analysis, leaves (three plants of *A. thaliana* or two leaves of tobacco for each sampling period) were harvested at different times after inoculation and stored at -80°C until further use. Total RNA was extracted following Suzuki *et al.* (2004) with some modifications. Briefly, leaves of randomly selected plants were ground in liquid nitrogen using a sterilized mortar and pestle and homogenized with the following extraction buffer: 100 mM Tris-HCl (pH 9.5), 10 mM EDTA (pH 8.0), 2% lithium dodecyl sulphate, 0.6 M NaCl, 0.4 M trisodium citrate and 5% 2-mercaptoethanol. Following centrifugation at room temperature, the resulting aqueous phase was re-extracted with a chloroform/isoamyl alcohol (24:1) mixture. The supernatant was collected and extracted with water-saturated phenol, guanidium thiocyanate, sodium acetate (pH 4.0) and chloroform. The upper aqueous phase was precipitated with isopropanol. The precipitated RNA was collected, washed, air-dried briefly and dissolved in RNase-free water. After treatment with RNase-free DNase, the DNase was inactivated according to the manufacturer's instructions (Takara Bio Inc.). Approximately 1 μg total RNA was reverse-transcribed into single-stranded cDNA using a mixture of oligo-dT primer, RNase inhibitor (20 U μL^{-1}) and RTase (50 U μL^{-1}) according to the manufacturer's instructions (Toyobo). An aliquot of the obtained cDNA was amplified by RT-PCR, as described by Suzuki *et al.* (2004), to monitor the expression of a set of well-characterized defence-related genes: *AtPR-1* (Pieterse *et al.*, 1998), *AtPR-2*, *AtHEL*, *AtPR-5* (Oñate-Sánchez & Singh, 2002), *AtPDF1.2*, *AtVSP* (Penninckx *et al.*, 1996), *AtActin* (Jones *et al.*, 2003), *NtPR-1a*, *NtNPR1*, *NtCoi1* (Wang *et al.*, 2009) and *NtActin* (Takabatake *et al.*, 2007). The gene-specific primers used in these experiments are listed in Table 1.

Data analysis

The data were subjected to analysis of variance (ANOVA) using XLSTAT PRO statistical analysis software (Addinsoft). The experiments were repeated at least three times, and treatment means were separated using a Fisher's least

significant difference (LSD) test. A Steel–Dwass test was conducted using EKUSERU-TOUKEI 2010 (Social Survey Research Information Co., Ltd). All analyses were conducted at a significance value of $P \leq 0.05$.

Results

Effect of BGI of *P. simplicissimum* GP17-2 on systemic protection against CMV in *A. thaliana* and tobacco

Symptoms of CMV appeared 10 dpi, and ranged from mild mosaic in young non-inoculated leaves to severe mosaic with stunting. At 14 dpi, *A. thaliana* and tobacco plants grown in soils amended with the BGI of GP17-2 exhibited a dramatic reduction in CMV symptoms as compared with the non-treated control plants, which showed severe symptoms of mosaic with small, deformed leaves (Fig. 1). All of the *A. thaliana* lines, *N. tabacum* cv. Xanthi-nc and *N. benthamiana* treated with the BGI of *P. simplicissimum* GP17-2 showed significant reductions in disease severity rating, similar to BTH-treated plants, 14 days after CMV inoculation (Fig. 2a,b).

Based on ELISA to measure CMV accumulation at 1 and 2 weeks after the virus challenge inoculation, CMV titre was significantly reduced in all *A. thaliana* and tobacco plants treated with BGI of GP17-2. However, the BTH-treated *npr1* transgenic plants showed no significant differences in CMV titre compared with the control plants (Fig. 3).

Effect of CF of *P. simplicissimum* GP17-2 on systemic protection against CMV

Two weeks after inoculation, the severity of CMV was significantly reduced in all *A. thaliana* and tobacco plants pre-treated with the CF of GP17-2 compared with the untreated control plants (Figs 4 and 5). Similarly, ELISA demonstrated that CMV titre was significantly reduced in *A. thaliana* and tobacco plants treated with CF of GP17-2 relative to the controls. These results confirm that the CF of GP17-2 was as effective as the BGI of GP17-2 (Fig. 6).

All of the *A. thaliana* lines had the same protection values, regardless of the GP17-2 inoculum type used, as did

Table 1 Gene-specific primers used in RT-PCR analysis in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>AtPR-1</i>	GTAGGTGCTCTTGTCTTCC	TTCACATAATTCCCACGAGG
<i>AtPR-2</i>	TCAAGGAAGGTTTCAGGGATG	TCGGTGATCCATTCTTCCACA
<i>AtHEL</i>	GTACCACCGCGGACACTGT	CAATGAGATGGCCTTGTGTA
<i>AtPR-5</i>	ATGGCAAATATCTCCAGTATTCACA	ATGTCGGGGCAAGCCGCGTTGAGG
<i>AtPDF1.2a</i>	AATGAGCTCTCATGGCTAAGTTTGCTTCC	AATCCATGGAATACACACGATTTAGCACC
<i>AtVSP</i>	TTTTACGCCAAAGGACTTGC	ATCCCGAGTTCCAAGAGGTT
<i>AtActin</i>	GTTGGGATGAACCAGAAGGA	GAACCACCGATCCAGACACT
<i>NtPR-1a</i>	GTGTAGAACCCTTGACCTGGGA	TTCGCCTCTATAATTACCTGGA
<i>NtNPR1</i>	GATGTGTGTTTTGTGTGGACAACGAGT	CCATCGGATGTCAGATCAGAAGGTCTAG
<i>NtCoi1</i>	GGATTGACTGATTTGGCGAAGG	TCCCTACTGGCTACAACCTCGT
<i>NtActin</i>	GGGTTTGTGGAGATGATGCT	GCTTCGTACCAACATATGCAT



Figure 1 Disease symptoms caused by *Cucumber mosaic virus* infection in (a) *Arabidopsis thaliana*, (b) *Nicotiana tabacum* and (c) *Nicotiana benthamiana* plants pre-treated with barley grain inoculum of *Penicillium simplicissimum* GP17-2, 2 weeks after virus challenge inoculation.

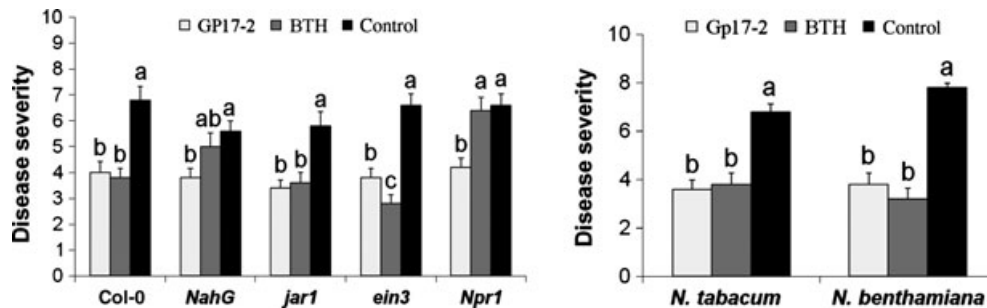


Figure 2 Disease severity in *Cucumber mosaic virus*-inoculated *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum* and *N. benthamiana*) plants treated with barley grain inoculum of *Penicillium simplicissimum* GP17-2 or with BTH, relative to non-treated control plants at 14 days post-inoculation. Columns represent mean values ($n = 10$, error d.f. = 27). Bars indicate standard errors. Different letters above columns indicate significant differences by the Steel–Dwass test for *A. thaliana* and Fisher's LSD test for tobacco ($P \leq 0.05$).

the tobacco plants. In addition, no significant differences were observed between the protection values induced by the BGI of GP17-2 or its CF in any *A. thaliana* lines or tobacco plants.

Effect of BGI of *P. simplicissimum* GP17-2 on the growth of *A. thaliana* and tobacco

Arabidopsis thaliana and tobacco plants treated with the BGI of GP17-2 exhibited a significant increase in shoot fresh weight and dry weight and number of leaves compared with the control plants (Table 2). The treated *A. thaliana* plants showed increases of 64%, 71% and approximately two leaflets in shoot fresh weight, shoot dry weight and number of leaves, respectively, over the control plants (Table 2). Similarly, treated *N. benthamiana* plants showed average increases of 105%, 90% and approximately one leaf in fresh weight, dry weight and number of leaves, respectively, compared with the control (Table 2). As for *N. tabacum*, plants exhibited 136% and 163% increases in average fresh weight and dry weight, respectively, compared with the control, but no significant increase in the number of leaves (Table 2). However, across all plants the BTH treatments resulted in significantly reduced growth compared with the control.

Root colonization

GP17-2 was reisolated at high frequencies from *A. thaliana* Col-0 and *N. tabacum* plants 6 weeks after

planting. The reisolation frequencies were 90% and 85% for *A. thaliana* Col-0 and *N. tabacum*, respectively.

Effect of BGI of *P. simplicissimum* GP17-2 on expression of SA- and JA/ET-inducible defence-related genes

For BGI-treated *A. thaliana* Col-0, the expression of *AtPR-1*, *AtPR-2*, *AtPR-5* and *AtHEL* was initially detected 1 day after CMV inoculation by RT-PCR and remained elevated at 6 dpi. Similarly, BTH-treated *A. thaliana* showed strong activation of PR genes prior to infection, and activity remained elevated at 6 dpi, whereas expression was not detected until 6 dpi in plants infected with CMV alone (control). By contrast, expression of *AtPDF1.2* was detected 2 dpi and appeared to decline at 4 dpi. Expression of *AtVSP* was detected 1 dpi and remained elevated at 2 dpi. The BTH treatment showed no stimulation of the JA/ET-responsive *AtPDF1.2* gene or the JA-responsive *AtVSP* gene after CMV infection. Similarly, none of the JA/ET-responsive genes showed enhanced expression after CMV infection in the control plants (Fig. 7).

In tobacco plants, expression of the *NtPR-1a* and *NtNPR1* genes was observed 1 day after the induction treatment and remained elevated for 6 dpi. The *NtCoi1* gene was elevated 1 dpi and appeared to decline 4 dpi (Fig. 8).

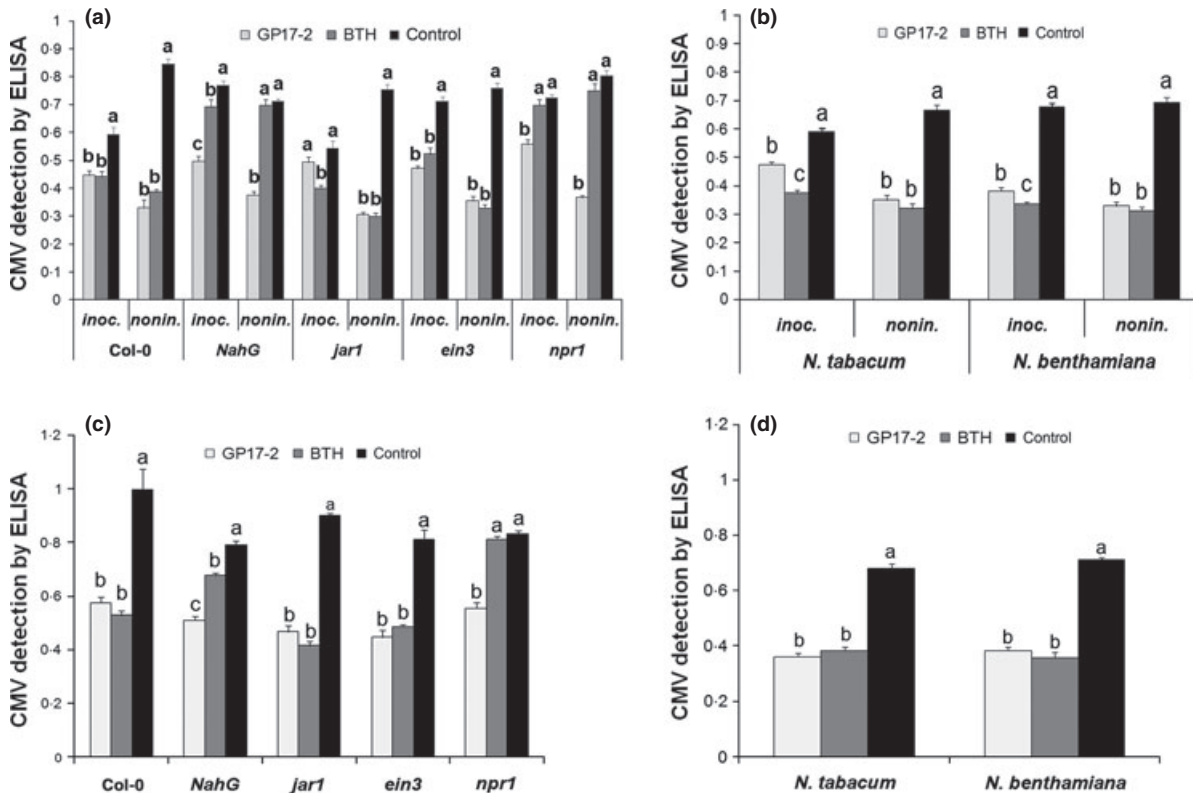


Figure 3 *Cucumber mosaic virus* accumulation in leaves of *Arabidopsis thaliana* lines, *Nicotiana tabacum* and *Nicotiana benthamiana* treated with either *Penicillium simplicissimum* GP17-2 or BTH at (a and b) 7 days and (c and d) 14 days post-inoculation. Columns represent mean values ($n = 4$, error d.f. = 9). Bars indicate standard errors. Statistical comparisons are among treatments within primary inoculated or secondary non-inoculated leaves and within the same line. Different letters indicate significant differences using Fisher's LSD ($P \leq 0.05$).



Figure 4 Disease symptoms caused by *Cucumber mosaic virus* infection of (a) *Arabidopsis thaliana*, (b) *Nicotiana tabacum* and (c) *Nicotiana benthamiana* plants pre-treated with culture filtrate of *Penicillium simplicissimum* GP17-2, 2 weeks after virus challenge inoculation.

Effect of CF of *P. simplicissimum* GP17-2 on expression of SA- and JA/ET-inducible defence-related genes

In *A. thaliana* plants, the CF of GP17-2 induced the expression of *AtPR-1*, *AtPR-2*, *AtPR-5*, *AtHEL* and *AtVSP* prior to inoculation, and expression remained elevated at 6 dpi. Similarly, BTH-treated *A. thaliana* showed elevated expression levels of *AtPR-1*, *AtPR-2*, *AtPR-5* and *AtHEL* after induction treatment, which remained elevated at 6 dpi. By contrast, none of the PR-responsive genes showed stimulated expression after CMV inoculation in the control plants. The expression of

AtPDF1.2 was best observed at 2 dpi in the CF treatment, while BTH and control treatments showed no stimulation of JA/ET-responsive genes after CMV infection (Fig. 9).

In tobacco plants, *NtPR-1a* and *NtNPR1* expression was detected 1 day after the induction treatment and remained elevated for 6 dpi. Expression of *NtCoi1* was initially detected 1 day after infection, remaining elevated at 6 dpi (Fig. 10).

Discussion

Induced systemic resistance is a widespread phenomenon that has been intensively investigated with respect to the

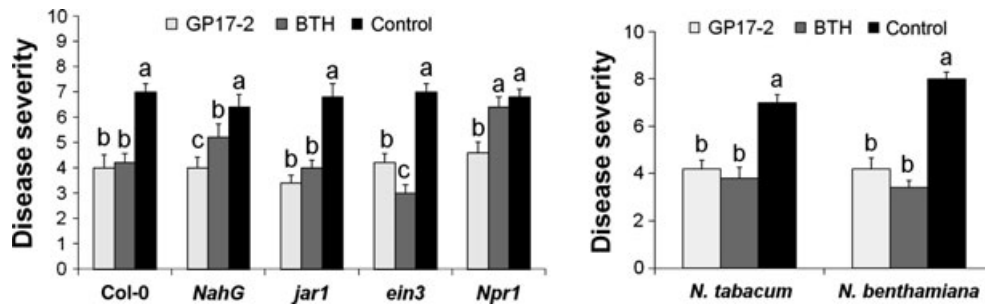


Figure 5 Disease severity of *Cucumber mosaic virus*-inoculated *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum* and *N. benthamiana*) plants treated with culture filtrate of *Penicillium simplicissimum* GP17-2 or with BTH, relative to non-treated control plants at 14 days post-inoculation. Columns represent mean values ($n = 10$, error d.f. = 27). Bars indicate standard errors. Different letters above columns indicate significant differences by the Steel–Dwass test for *A. thaliana* and Fisher's LSD test for tobacco at $P \leq 0.05$.

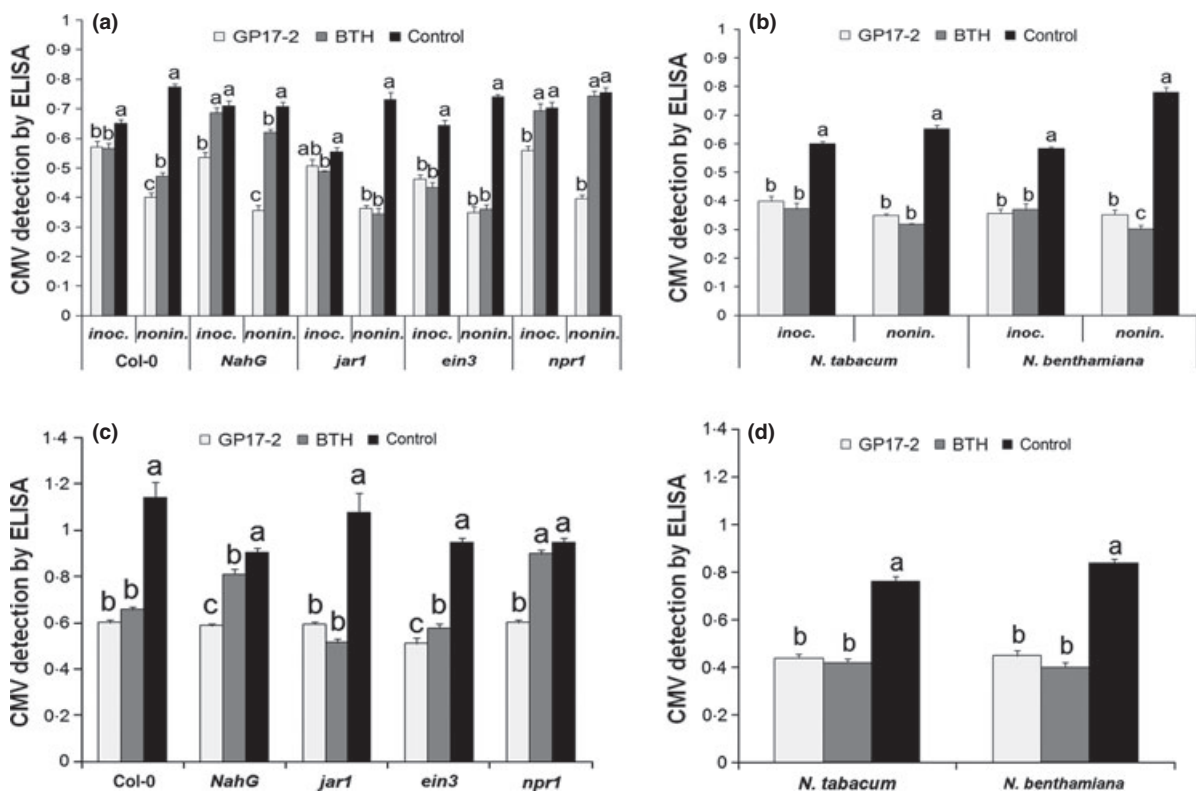


Figure 6 *Cucumber mosaic virus* accumulation in leaves of *Arabidopsis thaliana* lines, *Nicotiana tabacum* and *Nicotiana benthamiana* treated with either culture filtrate of *Penicillium simplicissimum* GP17-2 or BTH at (a and b) 7 days and (c and d) 14 days post-inoculation. Columns represent mean values ($n = 4$, error d.f. = 9). Bars indicate standard errors. Statistical comparisons are among treatments within inoculated or non-inoculated leaves and within the same line. Different letters indicate significant differences using Fisher's LSD ($P \leq 0.05$).

underlying signalling pathways and potential use in plant protection (Heil & Bostock, 2002). Several studies have reported resistance induction against local lesion viruses, but the inefficiency of the treatment against systemic invasion by viruses is of little value for controlling the disease (Di Piero *et al.*, 2010). Although many studies have shown the occurrence of viral inhibition by PGPR, mainly in studies of CMV with *A. thaliana* or tobacco plants (Ryu *et al.*, 2004, 2007; Ipper *et al.*, 2008; Thapa *et al.*,

2009; Wang *et al.*, 2009), little is known about the ISR against plant viruses associated with PGPF.

The results of this study demonstrated that the BGI and CF of GP17-2 could protect *A. thaliana* and tobacco plants against CMV, manifested as reductions in disease severity and in viral titre in leaves. Plants treated with a chemical inducer (BTH) of the plant defence response displayed responses that were similar, but not identical, to those of plants inoculated with GP17-2. Grohmann &

Table 2 Effect of *Penicillium simplicissimum* GP17-2 colonization and BTH treatment on growth of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Nicotiana benthamiana*

Treatment	Fresh weight			Dry weight			Number of leaves/plant		
	<i>A. thaliana</i>	<i>N. tabacum</i>	<i>N. benthamiana</i>	<i>A. thaliana</i>	<i>N. tabacum</i>	<i>N. benthamiana</i>	<i>A. thaliana</i>	<i>N. tabacum</i>	<i>N. benthamiana</i>
GP17-2	1.00 ± 0.05 ^{a*}	9.52 ± 0.38 ^a	5.38 ± 0.21 ^a	0.12 ± 0.01 ^a	1.13 ± 0.09 ^a	0.57 ± 0.02 ^a	18.80 ± 0.49 ^a	7.20 ± 0.49 ^a	7.60 ± 0.40 ^a
BTH	0.40 ± 0.01 ^b	2.76 ± 0.16 ^b	1.58 ± 0.12 ^b	0.05 ± 0.00 ^b	0.26 ± 0.02 ^b	0.17 ± 0.01 ^b	15.20 ± 0.49 ^b	6.40 ± 0.40 ^a	6.00 ± 0.00 ^b
Control	0.61 ± 0.03 ^c	4.04 ± 0.15 ^c	2.62 ± 0.11 ^c	0.07 ± 0.00 ^c	0.43 ± 0.02 ^c	0.30 ± 0.01 ^c	16.80 ± 0.49 ^c	6.80 ± 0.49 ^a	6.8 ± 0.49 ^{bc}

*Values are means ± SEM ($n = 5$, error d.f. = 12). Different letters indicate significant differences by Fisher's LSD at $P \leq 0.05$.

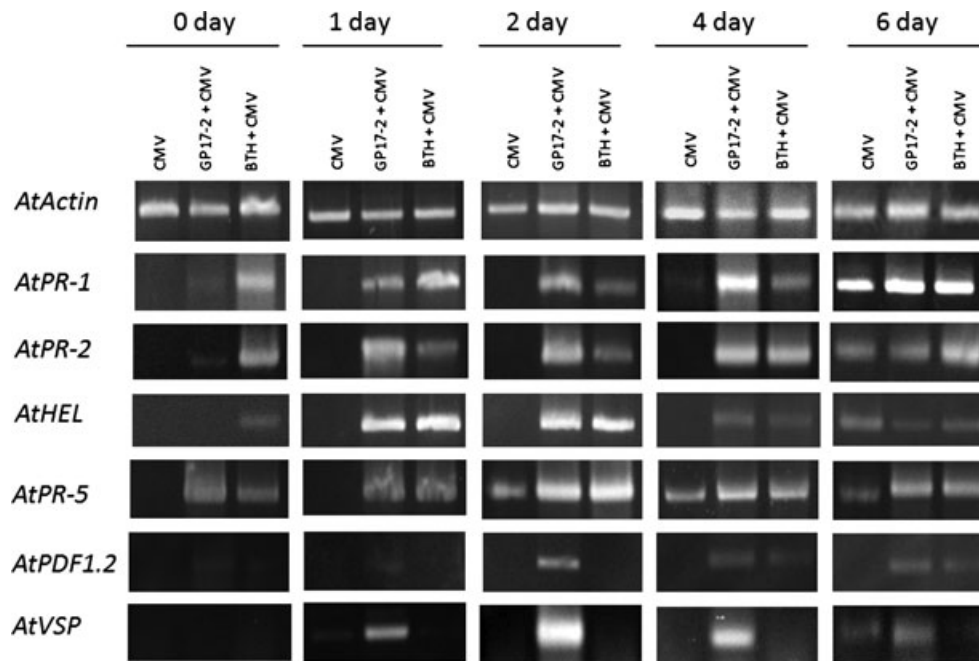


Figure 7 Expression of defence-related genes in leaves of *Arabidopsis thaliana* Col-0 plants treated with *Penicillium simplicissimum* GP17-2 or 0.3 mM BTH 1 day before challenge inoculation with *Cucumber mosaic virus*. SA-responsive (*AtPR-1*, *AtPR-2* and *AtPR-5*), ET/JA-responsive (*AtPDF1.2*), ET-responsive (*AtHEL*) and JA-responsive (*AtVSP*) genes were analysed as representative markers by RT-PCR using specific primers for each gene. A constitutively expressed *AtActin* was used as a control in RT-PCR.

Musumeci (1972) obtained similar results when they uncovered inhibitors to TMV infection in the culture filtrate from *Aspergillus flavus*. There was also a report that mycolaminaran, a β -1,3-glucan which was purified from *Phytophthora megasperma* cytoplasm, could reduce TMV incidence (Zinnen *et al.*, 1991). Mycolaminaran also inhibited the incidence of infection of *Datura stramonium* and *Nicotiana glutinosa* by four strains of *Cauliflower mosaic virus* (CaMV) and *Tomato spotted wilt virus* (TSWV), respectively (Heinkel *et al.*, 1992). The metabolites produced by the cyanobacteria *Synechococcus leopoliensis* and *Nostoc* sp. affected the incidence of TMV (Di Piero *et al.*, 2000).

This study used several *A. thaliana* genotypes to elucidate the signalling pathways involved in these responses. In the disease suppression experiment including both the BGI and CF of GP17-2, significant disease suppression was observed in *A. thaliana* plants expressing the *NahG* transgene which impairs SA accumulation, indicating

that the induction was independent of the SA signalling pathway. Similarly, *A. thaliana* mutants impaired in the JA (*jar1*)/ET (*ein2*) signalling pathways failed to disrupt the GP17-2-mediated suppression of CMV. *Npr1* is required for both SAR and ISR (Mou *et al.*, 2003); however, the *npr1* mutant showed full protection after treatments with the BGI and CF of GP17-2, indicating that the *npr1* mutation did not attenuate the GP17-2-mediated suppression of CMV.

There were strong correlations between disease severity and ELISA values in the different treatments in *A. thaliana* and tobacco plants. Moreover, *A. thaliana* and tobacco plants treated with either the BGI or CF of GP17-2 showed significantly lower ELISA values in non-inoculated leaves than in inoculated leaves at 7 and 14 dpi. These data indicate that treatment with the BGI or CF of GP17-2 limited virus movement into young leaves. Naylor *et al.* (1998) reported that the SA-mediated resistance in tobacco to *Potato virus Y* and

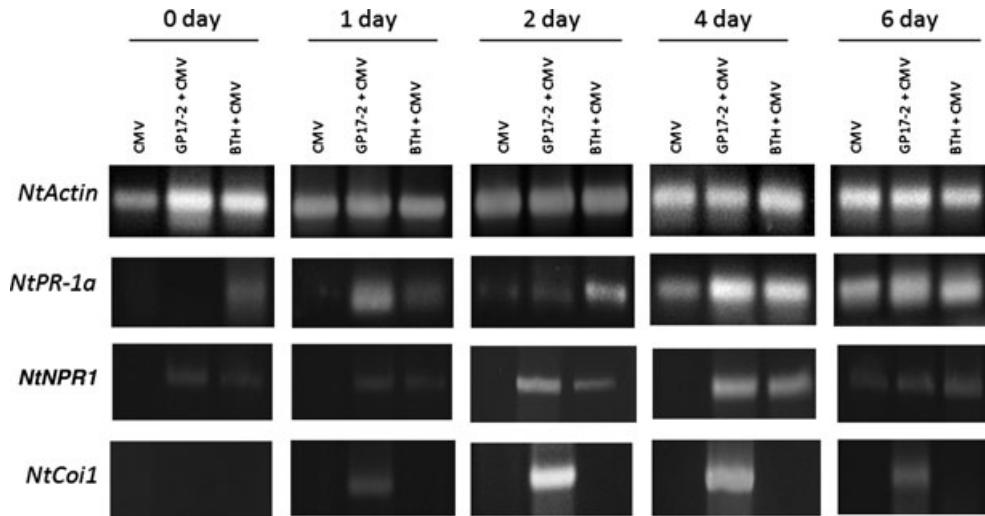


Figure 8 Expression of defence-related genes in leaves of *Nicotiana tabacum* plants treated with *Penicillium simplicissimum* GP17-2 or 0.3 mM BTH 1 day before challenge inoculation with *Cucumber mosaic virus*. SA-responsive *NtPR-1a*, a regulator of various defence genes (*NtNPR1*), and a regulator of the JA-dependent pathway (*NtCoi1*) were analysed as representative marker genes by RT-PCR using specific primers for each gene. A constitutively expressed *NtActin* was used as a control in RT-PCR.

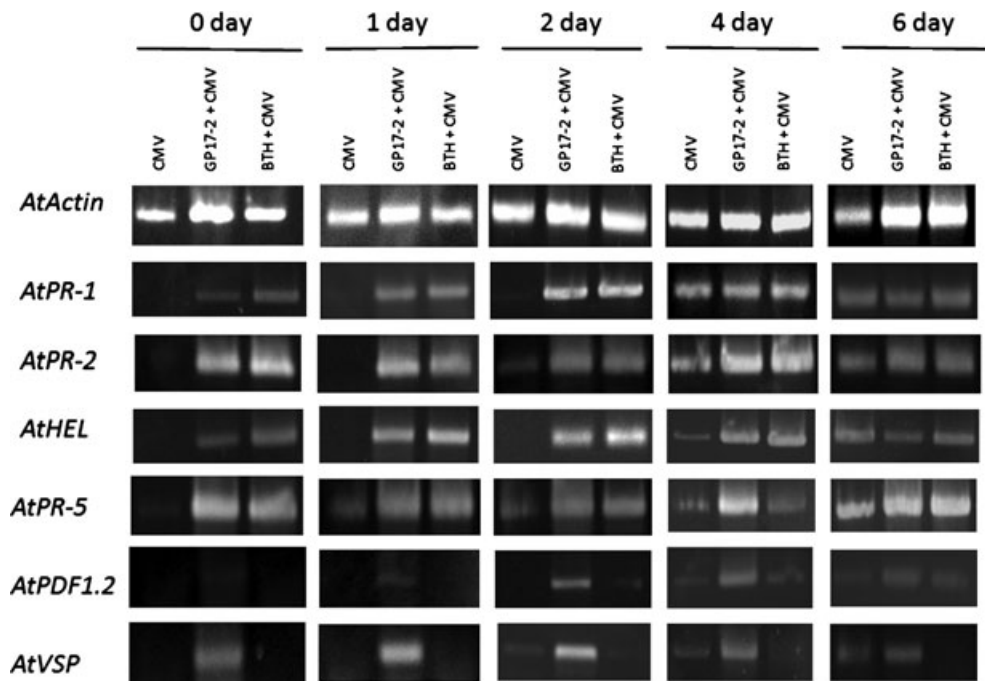


Figure 9 Expression of defence-related genes in leaves of *Arabidopsis thaliana* Col-0 plants treated with culture filtrate of *Penicillium simplicissimum* GP17-2 or 5 mM BTH 1 day before challenge inoculation with *Cucumber mosaic virus*. SA-responsive (*AtPR-1*, *AtPR-2* and *AtPR-5*), ET/JA-responsive (*AtPDF1.2*), ET-responsive (*AtHEL*) and JA-responsive (*AtVSP*) genes were analysed as representative markers by RT-PCR using specific primers for each gene. A constitutively expressed *AtActin* was used as a control in RT-PCR.

CMV was related to the inhibition of viral replication and movement, respectively.

In the present study, roots were treated with the BGI or CF of GP17-2, and CMV was inoculated into the leaves, so that no direct contact existed between the two micro-

organisms. The treatments with the BGI and CF of GP17-2 probably induced changes in the roots that extended up to the leaves and activated defence mechanisms. Therefore, this suppression of disease seems to be via the activation of induced systemic resistance. These results

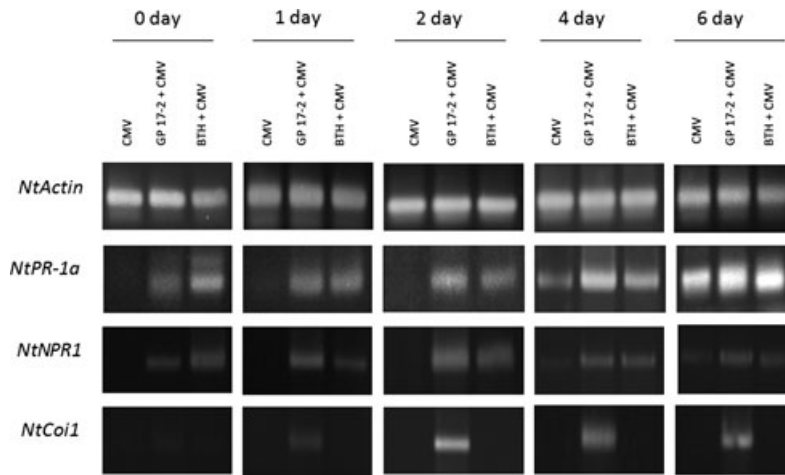


Figure 10 Expression of defence-related genes in leaves of *Nicotiana tabacum* plants treated with culture filtrate of *Penicillium simplicissimum* GP17-2 or 5 mmol L⁻¹ BTH 1 day before challenge inoculation with *Cucumber mosaic virus*. SA responsive *NtPR-1a*, a regulator of various defence genes (*NtNPR1*), and a regulator of JA-dependent pathway (*NtCoi1*) were analysed as representative marker genes by RT-PCR using specific primers for each gene. A constitutively expressed *NtActin* was used as a control in RT-PCR.

confirmed that GP17-2 and its CF activate different signalling pathways against bacterial and viral pathogens. The data also demonstrated that the BGI of *P. simplicissimum* GP17-2 enhanced the growth of *A. thaliana* and tobacco plants. These data are consistent with those obtained for the BGI of GP17-2 in *A. thaliana* plants by Hossain *et al.* (2007). Shivanna *et al.* (1994) reported that the ammonium-N content of barley grains colonized by certain PGPF isolates increased as a result of the increased root utilization of ammonium-N and subsequent enhanced plant growth. Mechanisms of growth promotion might be the ability of the fungus to provide minerals to plants in a more available form and the suppression of attacking pathogens. Also, PGPF could produce plant hormones (such as auxins, cytokinins or gibberellins), that alter root morphology and stimulate growth (Furukawa *et al.*, 1996; Contreras-Cornejo *et al.*, 2009).

Molecular and genomic tools are now being used to uncover the complexity of the induced defence-signalling networks that have evolved during the co-evolutionary arms race between plant defences and their attackers (Pieterse & Dicke, 2007). It has been demonstrated that a network of interconnected signal transduction pathways in which SA, JA and ET play central roles regulates plant defence responses (Robert-Seilantantz *et al.*, 2007). These signalling pathways do not function independently but influence each other through a complex network of synergistic and antagonistic interactions (Koornneef & Pieterse, 2008). The present study analysed the expression of the defence-related marker genes of the SA- and JA/ET-dependent pathways. Both the BGI and CF of GP17-2 up-regulated the expression of the SA-responsive PR genes *AtPR-1*, *AtPR-2*, *AtPR-5* and *NtPR-1a*, the JA/ET-responsive gene *AtPDF1.2*, the ET-responsive gene *AtHEL*, the JA-responsive gene *AtVSP*, the key player in activating the JA signalling pathway *NtCoi1*, and the

regulator of various sets of defence genes *NtNPR1*, compared with the challenged control plants. The BTH treatments in soil and rockwool systems showed an induction of SA-responsive genes and *AtHEL*, but not *AtPDF1.2* or *NtCoi1*. These results indicate that BTH can induce SA-responsive genes (Kohler *et al.*, 2002). *AtHEL* is an ET-responsive gene, and SA might mediate its induction in response to BTH treatment as reported by van Wees *et al.* (1999) and Norman-Setterblad *et al.* (2000). Previous studies reported that some PGPF and PGPR strains could activate the induction of defence genes in *A. thaliana* and tobacco plants. Hossain *et al.* (2007) found that expression of the defence *AtPR* and *AtVsp* genes in mutant plants after CF treatment was enhanced in only the *ein2* mutant; by contrast, *AtPDF1.2* was eliminated in *jar1* and *ein2* mutants. Additionally, treatment of tobacco plants with *Bacillus* spp. activated the induction of *NtPR1*, *NtNPR1* and *NtCoi1* genes, indicating that *Bacillus* spp. mediated ISR against viral infection (Wang *et al.*, 2009). Ahn *et al.* (2002) showed that treatment with selected strains of *Bacillus amyloliquefaciens* caused a rapid transcript accumulation of the defence-related genes, including *PR-1a*, phenylalanine ammonia-lyase (*PAL*), and 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) in tobacco plants after inoculation treatment. In addition, the treatment of *A. thaliana* plants resulted in the activation of *AtPR-1* and *AtPDF1.2*.

In conclusion, the data show that treatment with the BGI of GP17-2 enhanced the growth of *A. thaliana* and tobacco compared with the BTH and control plants. Additionally, the disease severity and ELISA data demonstrated that treatment with the BGI or CF of GP17-2 suppressed CMV in *A. thaliana* and tobacco plants. The RT-PCR analysis showed that treatment with the GP17-2 or its CF increased the expression of SA-, JA- and ET-inducible genes. These results indicate that GP17-2 and

its CF elicit ISR against CMV-Y via multiple pathways in *A. thaliana* and tobacco plants.

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References

- Ahn IP, Park K, Kim CH, 2002. Rhizobacteria-induced resistance perturbs viral disease progress and triggers defense-related gene expression. *Molecules and Cells* **13**, 302–8.
- Cao H, Bowling SA, Gordon AS, Dong X, 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *The Plant Cell* **6**, 1583–92.
- Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR, 1997. Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* **89**, 1133–44.
- Contreras-Cornejo HA, Macías-Rodríguez L, Cortés-Penagos C, López-Bucio J, 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. *Plant Physiology* **149**, 1579–92.
- von Dahl CC, Baldwin IT, 2007. Deciphering the role of ethylene in plant–herbivore interactions. *Journal of Plant Growth Regulation* **26**, 201–9.
- Di Piero RM, Pascholati SF, Rezende JAM, 2000. Efeito das cianobactérias *Synechococcus leopoliensis* e *Nostoc* sp. na infectividade do vírus do mosaico do fumo (TMV). *Summa Phytopathologica* **26**, 215–20.
- Di Piero RM, Novaes QS, Pascholati SF, 2010. Effect of *Agaricus brasiliensis* and *Lentinula edodes* mushrooms on the infection of passion flower with *Cowpea aphid-borne mosaic virus*. *Brazilian Archives of Biology and Technology* **53**, 269–78.
- Dong X, 1998. SA, JA, ethylene, and disease resistance in plants. *Current Opinion in Plant Biology* **1**, 316–23.
- Doombos RF, Geraats BPJ, Kuramae EE, van Loon LC, Bakker PAHM, 2011. Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions* **24**, 395–407.
- Fujiwara T, Hirai YM, Chino M, Komeda Y, Naito S, 1992. Effect of sulfur nutrition on expression of soybean seed storage protein genes in transgenic petunia. *Plant Physiology* **99**, 263–8.
- Furukawa T, Koga J, Adachi T, Kishi K, Syono K, 1996. Efficient conversion of L-tryptophan to indole-3-acetic acid and/or tryptophol by some species of *Rhizoctonia*. *Plant and Cell Physiology* **37**, 899–905.
- Görlach J, Volrath S, Knauf-Beiter G et al., 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *The Plant Cell* **8**, 629–43.
- Grohmann AA, Musumeci M, 1972. An inhibitor of *Tobacco mosaic virus* produced by *Aspergillus flavus*. *Arquivos do Instituto Biológico (Sao Paulo)* **39**, 69–73.
- Heil M, Bostock R, 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Annals of Botany* **89**, 503–12.
- Heinkel CM, Hudspeth MES, Meganathan R, Zinnem TM, 1992. Further characterization of mycolaminaran-induced resistance: temperature sensitivity against *Tobacco mosaic virus* and function against *Cauliflower mosaic virus* and *Tomato spotted wilt virus*. *Phytopathology* **82**, 637–41.
- Hossain MM, Sultana F, Kubota M, Koyama H, Hyakumachi M, 2007. The plant growth-promoting fungus *Penicillium simplicissimum* GP17-2 induces resistance in *Arabidopsis thaliana* by activation of multiple defense signals. *Plant and Cell Physiology* **48**, 1724–36.
- Hyakumachi M, 1994. Plant-growth-promoting fungi from turfgrass rhizosphere with potentials for disease suppression. *Soil Microorganisms* **44**, 53–68.
- Ipper NS, Lee SH, Suk JK et al., 2008. Antiviral activity of the expolysaccharide produced by *Serratia* sp. strain Gsm01 against *Cucumber mosaic virus*. *Journal of Microbiology and Biotechnology* **18**, 67–73.
- Jones AM, Ecker JR, Chen J-G, 2003. A reevaluation of the role of the heterotrimeric G protein in coupling light responses in *Arabidopsis*. *Plant Physiology* **131**, 1623–7.
- Kang SH, Cho HS, Cheong H, Ryu CM, Kim JF, Park SH, 2007. Two bacterial endophytes eliciting both plant growth promotion and plant defense on pepper (*Capsicum annuum* L.). *Journal of Microbiology and Biotechnology* **17**, 96–103.
- Kohler A, Schwindling S, Conrath U, 2002. Benzothiadiazole induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in *Arabidopsis*. *Plant Physiology* **128**, 1046–56.
- Koike N, Hyakumachi M, Kageyama K, Tsuyumu S, Doke N, 2001. Induction of systemic resistance in cucumber against several diseases by plant growth-promoting fungi: lignification and superoxide generation. *European Journal of Plant Pathology* **107**, 523–33.
- Koornneef A, Pieterse CMJ, 2008. Cross talk in defense signaling. *Plant Physiology* **146**, 839–44.
- Kunkel BN, Brooks DM, 2002. Cross talk between signalling pathways in pathogen defense. *Current Opinion in Plant Biology* **5**, 325–31.
- Lawton KA, Weymann K, Friedrich L, Vernooij B, Uknes S, Ryals J, 1995. Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Molecular Plant–Microbe Interactions* **8**, 863–70.
- Lawton KA, Friedrich L, Hunt M et al., 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *The Plant Journal* **10**, 71–82.
- Lee HJ, Park KH, Shim JH et al., 2005. Quantitative changes of plant defense enzymes in biocontrol of pepper (*Capsicum annuum* L.) late blight by antagonistic *Bacillus subtilis* HJ927. *Journal of Microbiology and Biotechnology* **15**, 1073–9.
- van Loon LC, Geraats BPJ, Linthorst HJM, 2006. Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science* **11**, 184–91.
- Lyon GD, Newton AC, 1997. Do resistance elicitors offer new opportunities in integrated disease control strategies? *Plant Pathology* **46**, 636–41.
- Martinez C, Blanc F, Le Claire E, Besnard O, Nicole M, Baccou JC, 2001. Salicylic acid and ethylene pathways are differentially

- activated in melon cotyledons by active or heat-denatured cellulase from *Trichoderma longibrachiatum*. *Plant Physiology* **127**, 334–44.
- Meera MS, Shivanna MB, Kageyama K, Hyakumachi M, 1995. Persistence of induced systemic resistance in cucumber in relation to root colonization by plant growth promoting fungal isolates. *Crop Protection* **14**, 123–30.
- Métraux J-P, Ahl-Goy P, Staub T *et al.*, 1991. Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In: Hennecke H, Verma DPS, eds. *Advances in Molecular Genetics of Plant–Microbe Interactions*; Vol. 1. Dordrecht, Netherlands: Kluwer Academic Publishers, 1.
- Mou Z, Fan W, Dong X, 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113**, 815–26.
- Naylor M, Murphy AM, Berry JO, Carr JP, 1998. Salicylic acid can induce resistance to plant virus movement. *Molecular Plant–Microbe Interactions* **11**, 860–8.
- Norman-Setterblad C, Vidal S, Palva ET, 2000. Interacting signal pathways control defense gene expression in *Arabidopsis* response to cell-wall degrading enzymes from *Erwinia carotovora*. *Molecular Plant–Microbe Interactions* **13**, 430–8.
- Oñate-Sánchez L, Singh BK, 2002. Identification of *Arabidopsis* ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. *Plant Physiology* **128**, 1313–22.
- Palukaitis P, Roossinck MJ, Dietzgen RG, Franki RIB, 1992. *Cucumber mosaic virus*. *Advances in Virus Research* **41**, 281–348.
- Penninckx IAMA, Eggermont K, Terras FRG *et al.*, 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *The Plant Cell* **8**, 2309–23.
- Pieterse CMJ, Dicke M, 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends in Plant Science* **12**, 564–9.
- Pieterse CMJ, van Wees SCM, van Pelt JA *et al.*, 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *The Plant Cell* **10**, 1571–80.
- Robert-Seilaniantz A, Navarro L, Bari R, Jones JD, 2007. Pathological hormone imbalances. *Current Opinion in Plant Biology* **10**, 372–9.
- Roossinck MJ, 1999. Cucumoviruses (Bromoviridae)—general features. In: Granoff L, Webster RG, eds. *Encyclopedia of Virology*, 2nd edn. San Diego, USA: Academic Press, 315–20.
- Ross AF, 1961. Systemic acquired resistance induced by localized virus infection in plants. *Virology* **14**, 340–58.
- Ryu C-M, Murphy JF, Mysore KS, Kloepper JW, 2004. 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. *The Plant Journal* **39**, 381–92.
- Ryu C-M, Kang BR, Han SH *et al.*, 2007. Tobacco cultivars vary in induction of systemic resistance against *Cucumber mosaic virus* and growth promotion by *Pseudomonas chlororaphis* O6 and its *gacS* mutant. *European Journal of Plant Pathology* **119**, 383–90.
- Schenk PM, Kazan K, Wilson I *et al.*, 2000. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proceedings of the National Academy of Sciences, USA* **97**, 11655–60.
- Shehata SF, El-Borollosy AM, 2008. Induction of resistance against *Zucchini yellow mosaic potyvirus* and growth enhancement of squash plants using some plant growth-promoting rhizobacteria. *Australian Journal of Basic and Applied Sciences* **2**, 174–82.
- Shivanna MB, Meera MS, Hyakumachi M, 1994. Sterile fungi from zoysiagrass rhizosphere as plant growth promoters in spring wheat. *Canadian Journal of Microbiology* **40**, 637–44.
- Shivanna MB, Meera MS, Hyakumachi M, 1996a. Role of root colonization ability of plant growth promoting fungi in suppression of take-all and common root rot of wheat. *Crop Protection* **15**, 497–504.
- Shivanna MB, Meera MS, Hyakumachi M, 1996b. Plant growth promoting fungi induced systemic resistance in cucumber. In: Wenhua T, Cook JM, Rovira A, eds. *Advances in Biological Control of Plant Diseases*. Beijing, China: China Agriculture University Press, 175–84.
- Shoman SA, Abd-Allah NA, El-Baz AF, 2003. Induction of resistance to *Tobacco necrosis virus* in bean plants by certain microbial isolates. *Egyptian Journal of Biology* **5**, 10–8.
- Staswick PE, Su W, Howell H, 1992. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Sciences, USA* **89**, 6837–40.
- Sticher L, Mauch-Mani B, Métraux J-P, 1997. Systemic acquired resistance. *Annual Review of Phytopathology* **35**, 235–70.
- Sultana F, Hossain MM, Kubota M, Hyakumachi M, 2009. Induction of systemic resistance in *Arabidopsis thaliana* in response to a culture filtrate from a plant growth-promoting fungus, *Phoma* sp. GS8-3. *Plant Biology* **11**, 97–104.
- Suzuki Y, Kawazu T, Koyama H, 2004. RNA isolation from siliques, dry seeds and other tissues of *Arabidopsis thaliana*. *BioTechniques* **37**, 542–4.
- Takabatake R, Karita E, Seo S, Mitsuhashi I, Kuchitsu K, Ohashi Y, 2007. Pathogen-induced calmodulin isoforms in basal resistance against bacterial and fungal pathogens in tobacco. *Plant and Cell Physiology* **48**, 414–23.
- Thapa SP, Lee HJ, Park DH *et al.*, 2009. Antiviral effects of the culture filtrate from *Serratia marcescens* Gsm01, against *Cucumber mosaic virus* (CMV). *The Plant Pathology Journal* **25**, 369–75.
- Thuerig B, Felix G, Binder A, Boller T, Tamm L, 2006. An extract of *Penicillium chrysogenum* elicits early defense-related responses and induces resistance in *Arabidopsis thaliana* independently of known signaling pathways. *Physiological and Molecular Plant Pathology* **67**, 180–93.
- Vallad GE, Goodman RM, 2004. Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science* **44**, 1920–34.
- Wang S, Wu H, Qiao J *et al.*, 2009. Molecular mechanism of plant growth promotion and induced systemic resistance to *Tobacco mosaic virus* by *Bacillus* spp. *Journal of Microbiology and Biotechnology* **19**, 1250–8.
- van Wees SCM, Luijendijk M, Smoorenburg I, van Loon LC, Pieterse CMJ, 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but

- stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. *Plant Molecular Biology* **41**, 537–49.
- Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG, 1998. *Coil1*: an *Arabidopsis* gene required for jasmonate regulated defense and fertility. *Science* **280**, 1091–4.
- Yan Z, Reddy MS, Ryu CM, McInroy JA, Wilson M, Kloepper JW, 2002. Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathology* **92**, 1329–33.
- Yarwood CE, 1960. Localized acquired resistance to tobacco mosaic virus. *Phytopathology* **50**, 741–4.
- Zehnder GW, Yao C, Murphy JF, Sikora ER, Kloepper JW, 2000. Induction of resistance in tomato against *Cucumber mosaic cucumovirus* by plant growth promoting rhizobacteria. *BioControl* **45**, 127–37.
- Zinnen TM, Heinkel CM, Hudpeth MES, Meganathan R, 1991. The role of cytoplasmic mycolaminaran in inhibiting initial viral infection of certain *Nicotiana* species. *Phytopathology* **81**, 426–8.