World J Microbiol Biotechnol (2016) 32:183
DOI 10.1007/s11274-016-2143-z

ORIGINAL PAPER

Characterization and evaluation of *Bacillus amyloliquefaciens* strain WF02 regarding its biocontrol activities and genetic responses against bacterial wilt in two different resistant tomato cultivars

Chu-Ning Huang[1] · Chan-Pin Lin[2,3] · Feng-Chia Hsieh[4] · Sook-Kuan Lee[3] · Kuan-Chen Cheng[3,5,6,7] · Chi-Te Liu[3,6]

Received: 11 December 2015 / Accepted: 13 September 2016 / Published online: 19 September 2016

Abstract *Bacillus amyloliquefaciens* strain WF02, isolated from soil collected at Wufeng Mountain, Taiwan, has siderophore-producing ability and in vitro antagonistic activity against bacterial wilt pathogen. To determine the impact of plant genotype on biocontrol effectiveness, we treated soil with this strain before infecting susceptible (L390) and moderately resistant (Micro-Tom) tomato cultivars with *Ralstonia solanacearum* strain Pss4. We also compared the efficacy of this strain with that of commercial *Bacillus subtilis* strain Y1336. Strain WF02 provided longer lasting protection against *R. solanacearum* than did strain Y1336 and controlled the development of wilt in both cultivars. To elucidate the genetic responses in these plants under WF02 treatment, we analyzed the temporal expression of defense-related genes in leaves. The salicylic acid pathway-related genes *phenylalanine ammonia-lyase* and *pathogenesis-related protein 1a* were up-regulated in both cultivars, whereas expression of the jasmonic acid pathway-related gene *lipoxygenase* was only elevated in the susceptible tomato cultivar (L390). These results suggest that WF02 can provide protection against bacterial wilt in tomato cultivars with different levels of disease resistance via direct and indirect modes of action.

Keywords Biocontrol · Bacterial wilt · *Bacillus amyloliquefaciens* · *Ralstonia solanacearum* · Induced plant resistance

Introduction

Bacterial wilt, a plant disease that occurs worldwide, is caused by the soil-borne phytopathogenic bacterium *Ralstonia solanacearum*, which can infect over 200 host species from 50 families, including several economically important crops such as tomato, potato, eggplant, banana and strawberry (Swanepoel 1992; Swanson et al. 2005).

Chu-Ning Huang and Chan-Pin Lin contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11274-016-2143-z) contains supplementary material, which is available to authorized users.

✉ Chi-Te Liu
chiteliu@ntu.edu.tw

[1] Master's Program for Plant Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan

[2] Department of Plant Pathology and Microbiology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan

[3] Institute of Biotechnology, National Taiwan University, R412., No. 81, Chang-Xing St., Taipei 106, Taiwan

[4] Biopesticides Division, Taiwan Agricultural Chemicals and Toxic Substances, Research Institute, Council of Agriculture, No. 11, Guangming Rd., Wufeng, Taichung 413, Taiwan

[5] Graduate Institute of Food Science and Technology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan

[6] Agricultural Biotechnology Research Center, Academia Sinica, No. 128 Sec. 2, Academia Rd., Nankang, Taipei 115, Taiwan

[7] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 404, Taiwan

This pathogen generally enters a plant via root colonization and then penetrates the xylem and systemically colonizes the stem, causing wilt symptoms (Buddenhagen and Kelman 1964). Conventional methods used for controlling bacterial wilt include chemical agents, resistant cultivars, crop rotation and culture practices (French and De Lindo 1982; Ghareeb et al. 2011; Michel and Mew 1998). Nonetheless, bacterial wilt is difficult to control because it can persist in moist environments and in host plant debris for long periods of time (Kulkarni and Patil 1982), and the methods applied are limited by location and climate factors (Vanitha et al. 2009). However, the use of beneficial organisms as biocontrol agents can serve as an environmentally friendly method of controlling this disease (Almoneafy et al. 2013; Lugtenberg and Kamilova 2009; Yamamoto et al. 2014).

Biocontrol agents of plant diseases are most often referred to as antagonists and include beneficial fungi and bacteria, bacteriophages, and avirulent pathogens that effectively suppress a wide spectrum of pathogens and reduce disease severity in many types of plants (Jogaiah et al. 2013; Ren et al. 2013; Vanitha et al. 2009; Yamamoto et al. 2014). However, the efficacy of biocontrol agents is usually influenced by the host plant genotype, and it is generally difficult to obtain the same efficiency with the same inoculant in different plant cultivars (Ryan et al. 2004).

The modes of action of biocontrol agents can be divided into direct and indirect mechanisms (Compant et al. 2005). Direct mechanisms include siderophore production, secretion of antimicrobial compounds or lytic enzymes, and detoxification (Compant et al. 2005). In contrast, indirect mechanisms include suppression of plant disease by biocontrol agents via the induction of plant resistance rather than direct inhibition of the pathogen. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance that are differentiated based on the nature of the elicitor and the regulatory pathways involved (Doornbos et al. 2012; van Loon et al. 1998).

Potent bacterial biocontrol agents include *Bacillus* spp., especially strains of *B. subtilis* and *Bacillus amyloliquefaciens*. These strains have been demonstrated to be effective for the biocontrol of bacterial wilt, *Fusarium* wilt, *Rhizoctonia* root rot, *Pythium* root rot, and other diseases (Chen et al. 2014; Gonzalez-Sanchez et al. 2013; Kim et al. 1997; Yamamoto et al. 2014). The biocontrol mechanisms involved include the production of antibiotics, siderophores, and hydrogen cyanide; competition for nutrients and space in the rhizosphere; induced resistance; and inactivation of the pathogen's enzymes (Choudhary and Johri 2009; Haas and Defago 2005; Raddadi et al. 2007). Furthermore, *Bacillus* spp. are suitable for commercialization because they produce heat- and desiccation-resistant spores, which can be readily formulated into stable products to provide protection from environmental stresses (Perez-Garcia et al. 2011).

In a preliminary study, *Bacillus* strain WF02 was selected for its high in vitro biocontrol efficacy against the bacterial wilt pathogen (Huang et al., unpublished data). To evaluate whether WF02 can be applied for protecting different plant genotypes from bacterial wilt disease, we used this bacterium to soil-inoculate a susceptible (L390) and a moderately resistant (Micro-Tom) tomato cultivar prior to infection with *R. solanacearum*. In addition, to evaluate the genetic responses of the different tomato cultivars, the temporal expression of defense-related genes was analyzed by real-time qPCR. A commercial *B. subtilis* strain, Y1336, was also used in this study for comparison.

Materials and methods

Bacterial materials

Several soil samples were collected from Wufeng mountain (Taichung county, Taiwan, 24°2′51.08″N, 120°42′48.57″E). To isolate *Bacillus* spp. strains, 5 g of each sample was suspended in 45 ml distilled water and heated to 60 °C for 30 min in a water bath. A 200-µl sample of the soil suspension was spread on a Luria–Bertani (Teitelbaum et al. 2007) agar plate for single-colony isolation, and approximately 40 isolates were selected. Strain WF02 was selected by in vitro screening for its high biocontrol efficacy against the bacterial wilt pathogen (Huang et al. unpublished data).

The commercial *B. subtilis* strain Y1336 (designated as B.s) derived from BIOBAC® WP (Bion-Tech, Taiwan), which is reported to be able to suppress bacterial wilt on tomato plants (Yang et al. 2012), was used as a control treatment. *R. solanacearum* Pss4 (race 1, biovar 3, designated as R.s), isolated from indigenous tomato, was provided by Dr. Chiu-Ping Cheng (Graduate Institute of Plant Biology, National Taiwan University).

Morphological characterization of bacterial isolates

WF02 and *B. subtilis* strain Y1336 (B.s) were cultured overnight in Luria–Bertani (Teitelbaum et al. 2007) broth at 28 °C and then subcultured as a 1 % bacterial suspension in LB broth for 4–5 h until the optical density (OD_{600}) reached 1.0 (approximately 10^8 CFU/ml). The morphology and Gram staining of vegetative cells was observed by optical microscopy (BH-51, Olympus, Tokyo, Japan), and single colonies were observed by stereomicroscopy (VEM-100, Optima, Taipei, Taiwan). To confirm virulence, *R.*

solanacearum Pss4 (R.s) was cultured on Kelman's tetrazolium chloride (TZC) agar medium (Kelman 1954) at 28 °C (Wang et al. 2000), i.e., the colonies are fluidal, irregular and creamy white with pink color at the center. This pathogen was then transferred to Casamino acid-Peptone-Glucose (CPG) medium (Granada and Sequeira 1983) and incubated for 48 h to prevent interference of the color during turbidity measurement of the culture using spectrophotometry. The bacterial cells were pelleted by centrifugation and suspended in sterile distilled water to adjust the number of cells in accordance with the requirement of each experiment described below.

Bacillus strain WF02 16S ribosomal RNA and *gyrB* gene and phylogenetic analyses

Bacterial genomic DNA was isolated from liquid culture using a Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan). 16S rDNA was amplified using the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT), and *gyrB* was amplified with GyrB-280F (GAAGTCATCATGACCGTTCTGCA) and 1730R (CCAAGACCTTTGTAACGCTG) (Eden et al. 1991; Yamada et al. 1999). The PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Chatsworth, CA, USA) and sequenced by the Center for Biotechnology at National Taiwan University. The gene sequences were compared to sequences in the GenBank database using Basic Local Alignment Search Tool (BLAST) (Borneman and Hartin 2000). Sequence alignment and analysis of gene similarity were performed using the MUSCLE program (Hall 2013). Evolutionary distances were calculated, and phylogenetic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987). The topology of the trees was evaluated by bootstrapping with 1000 resamplings (Felsenstein 1985). Phylogenetic trees were drawn using the MEGA6 program (Hall 2013). The accession numbers for bacterial strain WF02 generated in this study are AB918710 (16S rDNA) and AB936835 (*gyrB*).

In vitro antagonism test

Antagonists were confirmed using the filter paper method described by Nguyen and Ranamukhaarachchi (2010). The OD_{600} of the pathogen was adjusted to 0.3 (approximately 10^8 CFU/ml), and 100 µl of the suspension was spread onto CPG agar medium. A 20-µl aliquot of *Bacillus* bacterial suspension (approximately 10^8 CFU/ml) was added to a sterile paper disk (8 mm diameter) on CPG medium containing the dried pathogen suspension, and the diameter of the inhibition zone was determined after incubating at 28 °C for 2 days. Antagonistic ability was measured by the following formula: (diameter of inhibition zone − diameter of bacterial colony)/diameter of bacterial colony.

Siderophore production

The siderophore-producing ability of the bacteria was determined by the method proposed by Husen (2003). A 20-µl aliquot of bacterial suspension was added to a sterile paper disk (8 mm diameter) on chrome azurol S (CAS) agar medium (Munar-Vivas et al. 2010), and the diameters of the orange zone and bacterial colony were recorded after incubating at 28 °C for 7 days. Siderophore-producing ability was measured by the following formula: (diameter of orange hole − diameter of bacterial colony)/diameter of bacterial colony.

Biofilm formation assay

An assay for biofilm adherence to an abiotic surface was conducted with a modified crystal violet staining method, as described by Zhao et al. (2015). The WF02 or B.s bacterial suspension was adjusted to $OD_{600} = 0.01$ in LB broth; 100 µl of the bacterial culture was added to a polystyrene 96-well plate and incubated at 28 °C. After 48 h, the culture was stained with 1 % crystal violet for 20 min and rinsed three times with water. The biofilm was dissolved with EtOH/acetone (80:20), and the OD_{570} value was determined by spectrophotometry.

Tomato pot experiments to test the in vivo effect of bacterial strain WF02 against *R. solanacearum*

Two tomato (*Solanum lycopersicum*) cultivars, cv. L390 (susceptible) and cv. Micro-Tom (moderately resistant), were used as host plants. Seeds were surface-sterilized with 70 % ethanol for 30 s followed by 1 % sodium hypochlorite solution for 15 min. The seeds were then washed three times with sterilized water for 1 min. After growing in a seed tray (1 × 1 × 1.5 cm) for 5 days, the tomato seedlings were transplanted into a 3-in. pot (one seed per pot) containing 90 g of sterilized organic soil with vermiculite (1:1). Cultivar L390 was incubated at 25 °C with a 12-h light period (approximately 4000 lux) in a growth chamber, as described by Chen et al. (2009b), whereas Micro-Tom was grown in a 30 °C growth chamber with a 12-h light period based on a preliminary test showing that the infection rate was higher at 30 °C than at 25 °C (Huang et al., unpublished data).

The following treatments for the pot experiments were used to determine the in vivo biocontrol efficacy of the *Bacillus* strains: (1) control 1 (designated R.s), inoculation of the pathogen (*R. solanacearum* Pss4) suspension only; (2) control 2 (B.s), inoculation of the commercial inoculant

(*B. subtilis* strain Y1336) only; (3) B.s + R.s, treatment with the *B. subtilis* Y1336 inoculant and the pathogen Pss4 suspension; (4) WF02 + R.s, treatment with the WF02 inoculant and the pathogen Pss4 suspension.

Initially, we pre-inoculated 5-week-old tomato seedlings with the respective bacterial inoculants (*B. subtilis* Y1336 or WF02) by drenching each pot, as described by Ramesh et al. (2009). The inoculation dosage was equivalent to 10^7 CFU/g soil. After 1 week, we poured the *R. solanacearum* Pss4 suspension into the soil. In consideration of the disease progression, the dosage was equivalent to 10^7 and 10^8 CFU/g soil for susceptible cultivar L390 and the moderately resistant cultivar Micro-Tom tomato, respectively. These experiments were repeated at least three times, with a sample size for each treatment of 8–10 plants.

Ralstonia solanacearum induced symptoms of wilting in the leaves and stems of individual tomato plants, and these symptoms were monitored for approximately 2 weeks after *R. solanacearum* inoculation. Disease severity was scored by a 5-level disease index (Wang et al. 2000), as follows: 0 = healthy; 1 = partial wilting of 1 lower leaf; 2 = wilting of 2–3 lower leaves and stems; 3 = wilting of all but 2–3 upper leaves and stems; 4 = wilting of all leaves and stems; 5 = plant death. The number of dead plants was recorded at 14 days post-pathogen inoculation (dpi). Disease severity was calculated by mortality ([the number of dead plants/total number of plants] × 100 %) and the area under the disease progress curve (AUDPC) (Almoneafy et al. 2013; Wang et al. 2000). AUDPC was calculated using the following formula:

$$\Sigma[Y_{i+1} + Y_i]/2 \times [X_{i+1} - X_i]$$

where Y_i = the percentage of disease incidence (%) at the ith observation, with i = 1 being the first observation point at time zero; X_i = the time (days) at the ith observation.

Detection of *R. solanacearum* Pss4 populations in tomato-cultivated soils via most-probable-number PCR

The most-probable-number PCR (MPN-PCR) method was used to detect and enumerate *R. solanacearum* Pss4 in rhizosphere soils (Fredslund et al. 2001; Svercel et al. 2010). Soil samples were collected from the 3-in. pots cultivated with L390 at 0, 1, 5, 10 and 15 dpi and with Micro-Tom at 0, 1, 7 and 14 dpi. Visible root fragments were removed from the samples. Total genomic DNA was extracted from 0.3 g of wet soil using the FASTDNA SPIN kit for soil (MP Biomedicals, Irvine, CA, USA) following the manufacturer's instructions. The specific primers AU759 (GTCGCCGTCAACTCACTTTCC) and AU750 (GTCGCCGTCAGCAATGCGGAATC) were used to amplify 282 bp of the putative *lpxC* gene of *R. solanacearum*. Total genomic DNA was then diluted to 10 ng/μl, and tenfold serial dilutions of the DNA sample were used as the MPN 3-tube dilution DNA templates (Marian et al. 2012). MPN-PCR was carried out in a 20-μl reaction mixture containing 10 μl 2 × *Taq* DNA polymerase master mix red (Ampliqon), 0.2 μl each primer, and 10 μl DNA template. Three replicates were used for each diluted sample to calculate MPN according to the following formula described by Thomas (1942):

$$\mathrm{MPN/g} = \left[\sum G_j\right] \Big/ \left\{\sum T_j M_j \times \left[\sum (T_j - G_j) M_j\right]\right\}^{(1/2)}$$

where G_j = the number of positive tubes in the jth dilution, with j = 1 being the first dilution of the sample; T_jM_j = the mass (in g) of samples in all tubes; and $(T_j - G_j)$ M_j = the mass (in g) of samples in all negative tubes.

Expression of plant defense-related genes

To analyze the expression of defense-related genes in the host plants, 0.1 g of leaf tissue was collected from the third true leaf from the top of each treated tomato plant at 0, 24, 48 and 72 h after pathogen inoculation. The samples were homogenized in liquid nitrogen four times for 5 s each using a tissue grinder (SH-100, Kurabo, Tokyo, Japan) at 6000 rpm. One milliliter of TriZol® (Invitrogen, Carlsbad, CA, USA) was then added to each sample, and the samples were stored at −80 °C until RNA extraction. Total RNA was extracted using a Direct-zol™ RNA Mini Prep kit (Zymo Research, Orange, CA, USA) following the manufacturer's protocol, and the RNA sample was eluted in 50 μl of elution buffer. The TURBO DNA-free™ kit (Invitrogen, Carlsbad, CA, USA) was used to eliminate any DNA contamination. RNA concentrations were measured using a Nanodrop ND1000 (Thermo Scientific, Wilmington, DE, USA) and quantified by RNA gel electrophoresis with ImageJ software (Schneider et al. 2012).

The expression patterns of the plant defense genes phenylalanine ammonia-lyase (*PAL*), pathogenesis-related gene (*PR1a*), lipoxygenase (*LOX*), and allene oxide cyclase (*ACO*), which are related to the SA, JA and ET signaling pathways, were analyzed by real-time PCR. Reverse transcription of 500 ng of total RNA was performed with Superscript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using oligo $(dT)_{20}$ primers. Real-time PCR was performed by adding 5 μl SYBR green mix (KAPA Biosystem, Woburn, MA, USA), 0.4 μl specific primers (10 mM) (Table 1) and 10 ng cDNA as a template in a total reaction volume of 10 μl. The program was as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. The melting curve was systematically checked at the end of each real-time PCR.

Table 1 Primers used in the qPCR analysis of tomato defense-related genes

Primer name	Sequence (5′–3′)	Target gene	Pathway[a]	References
PAL-F	TTCGAGTTGCAGCCTAAGGAAGGA	Phenylalanine ammonia-lyase	SA	Wu et al. (2012)
PAL-R	ATAGCAGCAGCCTCAATCTGACCA	Phenylalanine ammonia-lyase	SA	Wu et al. (2012)
PR1a-F	GAGGGCAGCCGTGCAA	Pathogenesis relative gene 1a	SA	Milling et al. (2011)
PR1a-R	CACATTTTTCCACCAACACATTG	Pathogenesis relative gene 1a	SA	Milling et al. (2011)
LOX-F	TTTCTGCGACTTGAGGTTCGG	Lipoxygenase	JA	Vanitha and Umesha (2011)
LOX-R	ATTAGTCTTTACCTTCTTGTCCAGT	Lipoxygenase	JA	Vanitha and Umesha (2011)
ACO-F	AGTGGCCTTCAACTCCTCAA	ACC oxidase	ET	This study
ACO-R	CGAGTCCCATCTGTTTGTGC	ACC oxidase	ET	This study
Actin-F	TCAGCAACTGGGATGATATG	Actin	HK	Milling et al. (2011)
Actin-R	TTAGGGTTGAGAGGTGCTTC	Actin	HK	Milling et al. (2011)

[a] Gene representative of the signaling pathway: *SA* salicylic acid, *JA* jasmonic acid, *ET* ethylene, *HK* housekeeping gene for normalization

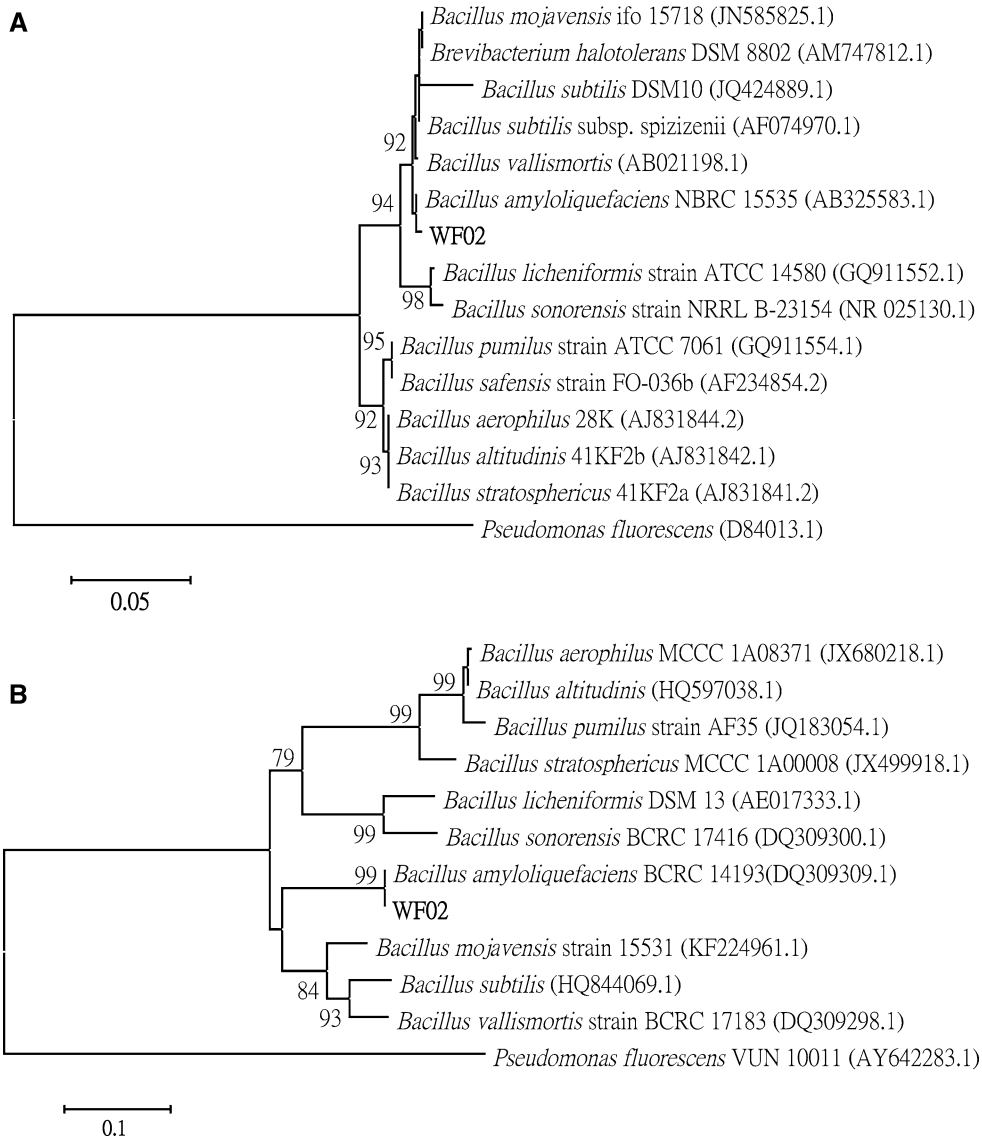

Fig. 1 Phylogenetic trees based on either the **a** 16S rRNA gene sequence or **b** *gyrB* gene sequence showing the relationships between WF02 and *Bacillus* species using the neighbor-joining method. The accession numbers for the sequences are given in parentheses after the strain names. *Numbers* at the nodes are the bootstrap values. The *scale bars* indicate the numbers of substitutions per nucleotide position, which were **a** 0.05 and **b** 0.1. The corresponding sequence of *Pseudomonas fluorescens* was used as an outgroup to root the 16S rDNA or *gyrB* tree

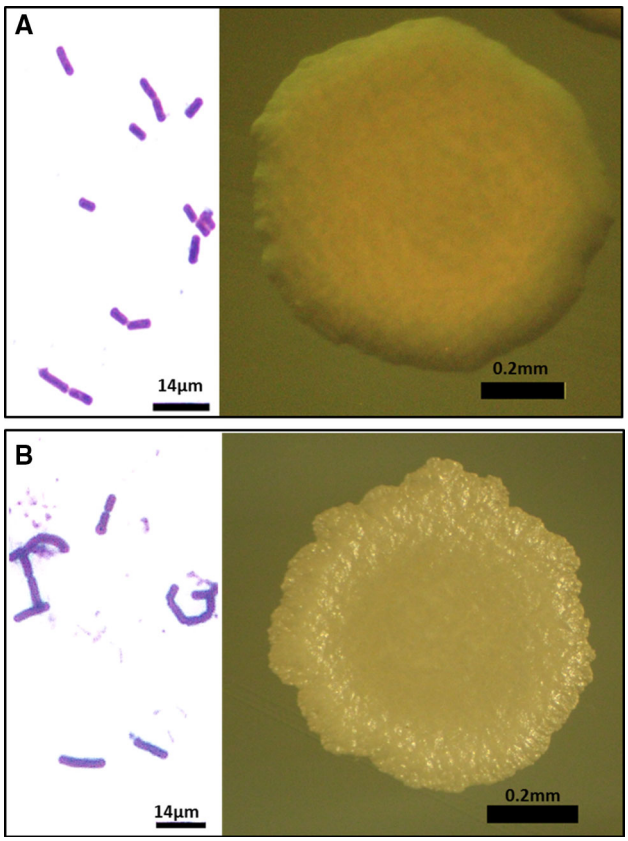

Fig. 2 Morphology of strain WF02 and *Bacillus subtilis* strain Y1336 (B.s). **a** WF02, **b** Y1336. The *left panel* shows the bright-field image under optical microscopy after Gram staining. The *right panel* shows a colony of the respective strain under stereomicroscopy

The relative expression levels of target gene transcripts were quantified using the Delta Delta CT (ΔΔCT) method according to the following equation (Milling et al. 2011):

$$\begin{aligned}\Delta\Delta\mathrm{CT} = &[(\mathrm{CT}(\text{target, treated}) - \mathrm{CT}(\text{ref, treated})]\\ &-[\mathrm{CT}(\text{target gene, untreated sample})\\ &- \mathrm{CT}(\text{ref gene, untreated})]\end{aligned}$$

where CT (target, untreated) is the CT value of gene of interest in the untreated sample (i.e., the control sample without treating with any inoculant B.s/WF02 or pathogen R.s), CT (ref gene, untreated) is the CT value of the housekeeping gene *Actin* (BT013524) in the untreated sample, CT (target, treated) is the CT value of gene of interest in respective treated sample (R.s, B.s + R.s, or WF02 + R.s), and CT (ref, treated) is the CT value of the housekeeping gene *Actin* (BT013524) in the treated sample.

We calculated the ratio of the respective target gene in the treated samples relative to the untreated sample using $2^{\Delta\Delta CT}$.

Three biological replicates were included in each treatment, and each experiment was repeated at least three times.

Statistical analysis

The in vivo biocontrol activity experiment and the in vitro bioassay experiments were analyzed by Fisher's least significant difference (LSD) multiple comparisons analysis at a significance level of $P = 0.05$. Statistical analysis for the quantification of bacteria was performed with one-way ANOVA, and the means were subjected to Duncan's multiple range tests at a significance level of $P = 0.05$.

Results

Identification and characterization of bacterial strain WF02

For phylogenetic assignment, the 16S rRNA sequence of the WF02 isolate was determined. This bacterium was identified as a *Bacillus* sp. by BLAST searching the 16S rRNA sequence against the GenBank database, and it was found to cluster with such bacteria as *B. amyloliquefaciens*, *B. subtilis*, and *B. vallismortis* in the 16S rRNA phylogenetic tree (Fig. 1a). The housekeeping gene *gyrB* of *Bacillus* spp. was used to discriminate the phylogenetic relationship among members of the genus *Bacillus* (Wang et al. 2007). As shown in Fig. 1b, strain WF02 clustered closely with *B. amyloliquefaciens* strain BCRC14193 (100 % *gyrB* gene sequence similarity). The cellular and colony morphologies of the WF02 isolate were observed by optical microscopy, and the results are shown in Fig. 2. We used the commercial inoculant *B. subtilis* strain Y1336 (B.s) as a reference for comparative analyses in this study. The cells of both strains are gram positive, motile, and rod-shaped, approximately 2.0 μm in length. Colonies of WF02 developing on LB agar under aerobic growth conditions are white to cream-colored, with a convex elevation, an undulate margin and are mucoid in appearance, as typical morphological characteristics of *B. amyloliquefaciens*. In contrast, colonies of Y1336 are cream-light yellow in color, with a concave elevation, an undulate margin and are mucoid in appearance.

In vitro assays for antagonism, siderophore production, and biofilm formation

Dual-culture assays were performed to confirm the in vitro antagonistic effect of WF02 against the growth of *R. solanacearum* Pss4 (R.s). We determined the inhibition zone and found that WF02 grew vigorously, inhibiting the growth of R.s after 24 h of incubation (Fig. 3a). There was no significant difference between WF02 and the commercial *B. subtilis* strain Y1336 (B.s) in this regard.

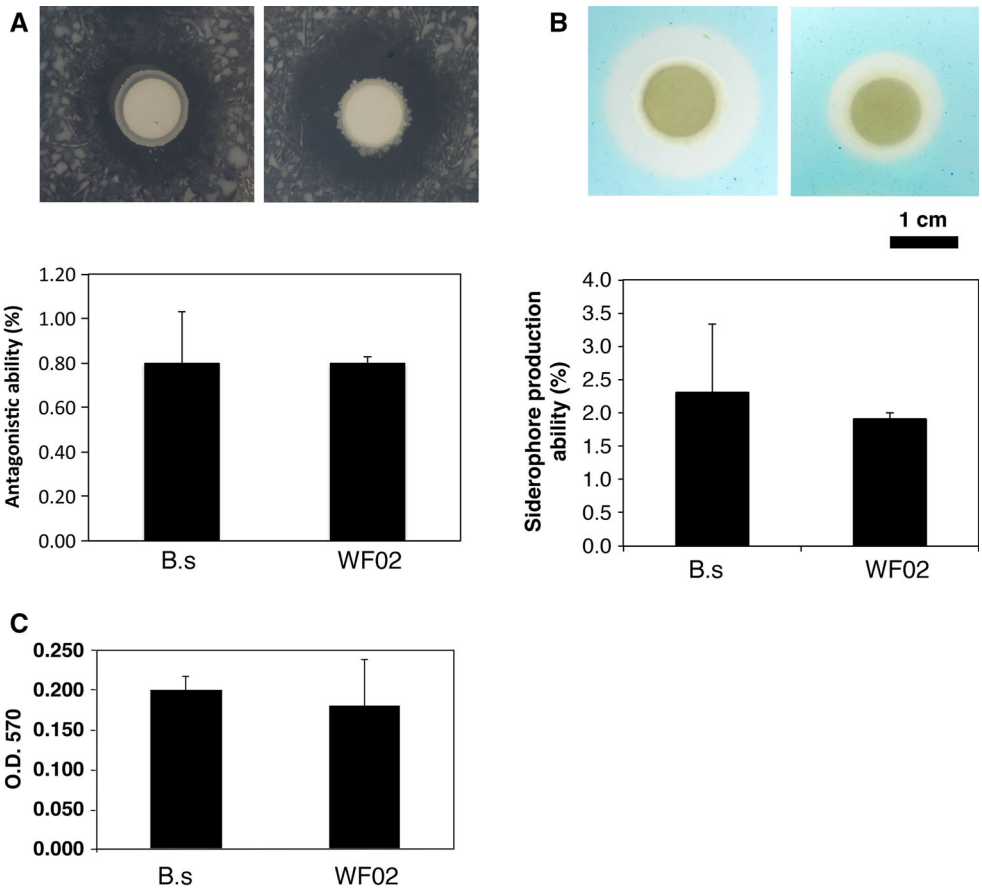

Fig. 3 In vitro antagonistic activity, siderophore-producing ability and colonization test of strain WF02 and *B. subtilis* strain Y1336 (B.s). **a** The antagonistic activity of the bacteria was evaluated against *R. solanacearum* Pss4 (R.s) by applying the dual culture method using CPG agar. **b** The siderophore-producing ability of the bacteria was determined using CAS agar. **c** Quantitative spectrophotometric analysis of the biofilm synthesized using the crystal violet staining method in LB medium; the OD_{570} value was determined by spectrophotometry

In addition, we conducted a blue agar CAS assay to verify the siderophore-producing ability of WF02. After several days of incubation, the blue color of the CAS medium disappeared, and a bright orange halo formed around the paper disc (Fig. 3b). This result suggests that WF02 can synthesize siderophores to chelate ferric ions, and this ability was similar to that of B.s.

We used a crystal violet staining assay to assess the ability of the two *Bacillus* strains (WF02 and B.s) to form biofilm on an abiotic surface. As shown in Fig. 3c, these two *Bacillus* strains exhibited similar capacities for biofilm adherence to a polystyrene surface.

Strain WF02 reduced the severity of bacterial wilt disease

To evaluate whether WF02 can be applied for protecting different plant genotypes from bacterial wilt disease, we used this bacterium to soil-inoculate a susceptible (L390) and a moderately resistant (Micro-Tom) tomato cultivar prior to infection with *R. solanacearum*. In a preliminary test, we evaluated the infection rate of R.s in different resistant tomato cultivars. The R.s pathogen did not cause any disease symptom when the moderately resistant Micro-Tom plants were grown at 25 °C, but it did result in disease development at 30 °C (data not shown). Conversely, at 30 °C, all of the L390 plants were dead within a very short time period after inoculation of the R.s pathogen; the suitable cultivation temperature was 25 °C. Accordingly, we cultivated L390 and Micro-Tom under different temperatures (25 and 30 °C, respectively), though it is unclear whether the colonization ability of the inoculants varies at different temperatures.

The disease severity of bacterial wilt can be determined by indices such as mortality and AUDPC (Almoneafy et al. 2013). When treating moderately resistant Micro-Tom with B.s in a pot before *R. solanacearum* Pss4 (R.s) infection (B.s + R.s), mortality was significantly reduced compared to that of the pathogen control (R.s) (from 16 to 4 %); although no statistical significant difference was found, the AUDPC was also lower than that for R.s (Fig. 4a). However, when we substituted WF02 for the B.s inoculant (WF02 + R.s), both mortality (from 16 to 3 %) and the AUDPC (233-92) were strikingly lower than in the R.s control treatment.

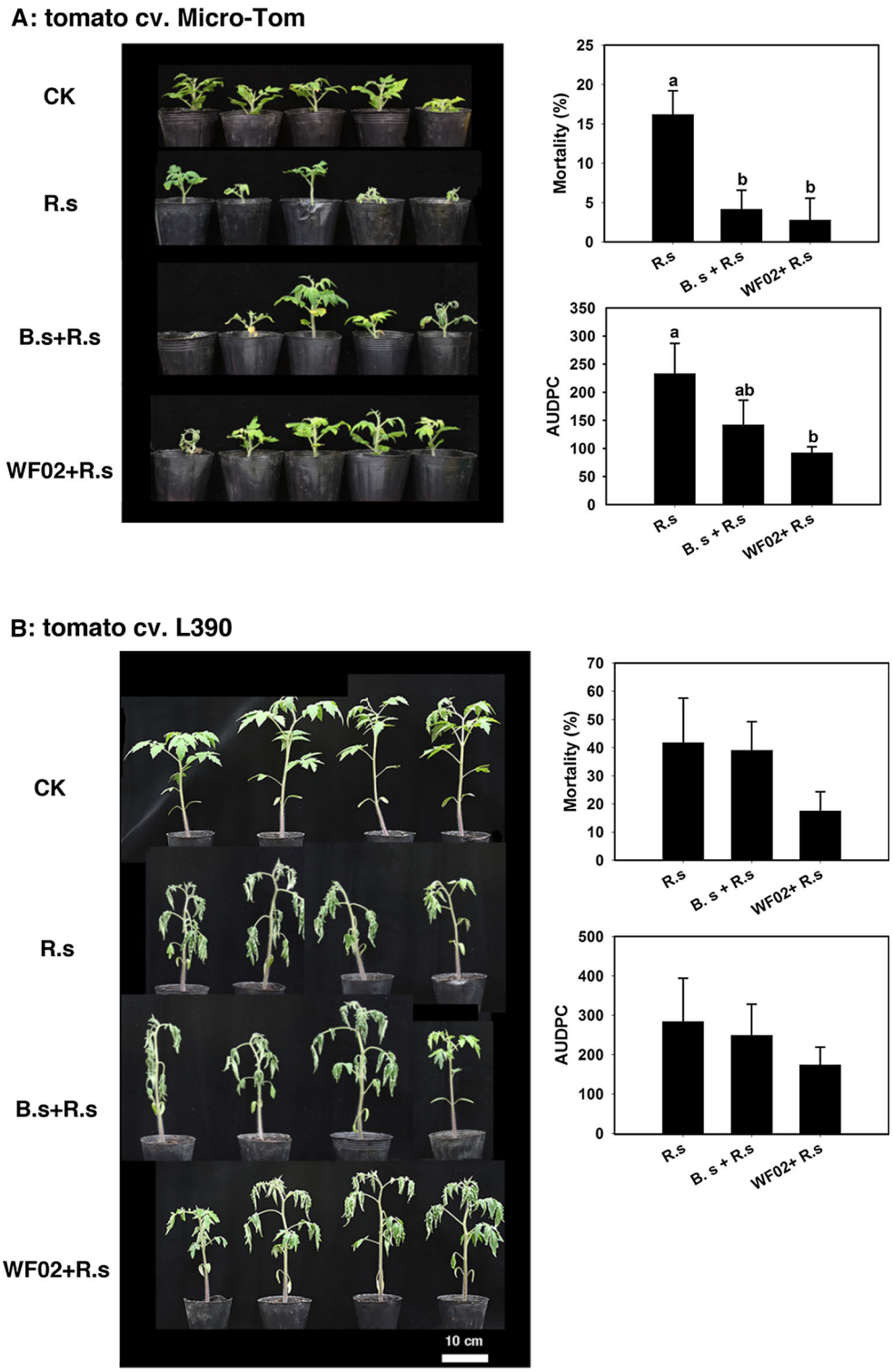

Fig. 4 Biocontrol activities of strain WF02 and *B. subtilis* strain Y1336 (B.s) against *R. solanacearum* in **a** moderately resistant Micro-Tom and **b** susceptible L390 tomato cultivars. The disease severity of bacterial wilt can be determined by mortality and the area under the disease progress curve (AUDPC). The data are presented as the mean ± SE. Locations marked with *different letters* (*a*, *b*) are significantly different by Fisher's least significant difference (LSD) multiple comparisons analysis ($P < 0.05$). *R.s* indicates the *R. solanacearum* treatment, *B.s* or *WF02 + R.s* indicates treatment with *B. subtilis* Y1336 or WF02 before *R. solanacearum* inoculation

In pot experiments using the susceptible cultivar L390, the WF02 + R.s treatment showed relatively low values of mortality (18 %) and AUDPC (174) in comparison with the R.s treatment (42 % and 284, respectively), although there was no statistically significant difference (Fig. 4b). Conversely, the B.s + R.s treatment failed to reduce the disease severity.

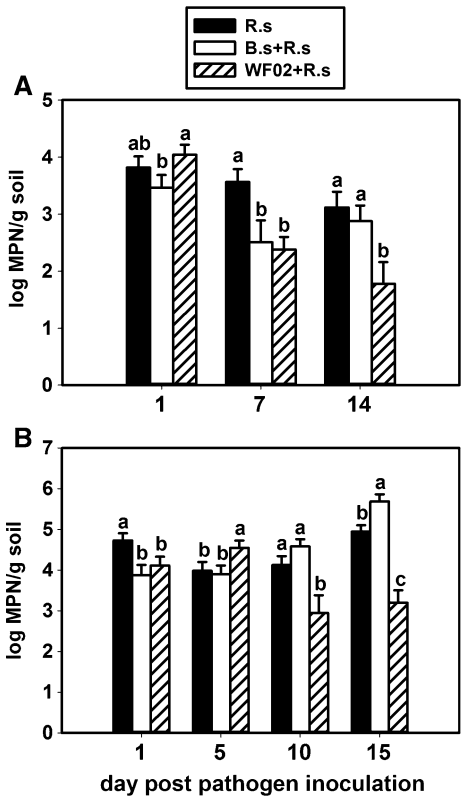

Fig. 5 Populations of *R. solanacearum* in tomato-cultivated soils over time. Soil samples were collected at 1, 7 and 14 dpi for pot experiments using Micro-Tom (**a**) and at 1, 5, 10 and 15 dpi for experiments using L390 (**b**). The data are presented as the mean ± SE. Locations marked with *different letters* (*a–c*) are significantly different by Fisher's least significant difference (LSD) multiple comparisons analysis ($P < 0.05$). *R.s* indicates the *R. solanacearum* treatment, *B.s* or *WF02 + R.s* indicates treatment with *B. subtilis* Y1336 or individual isolates before *R. solanacearum* inoculation

Populations of *R. solanacearum* over time in tomato-cultivated soils

We used MPN-PCR to enumerate the remaining populations of *R. solanacearum* Pss4 over time in post-harvested soils. In the pot experiments with moderately resistant Micro-Tom (Fig. 5a), the population of *R. solanacearum* (R.s) was significantly decreased at 7 dpi under pretreatment with either the B.s or the WF02 isolate. In contrast, B.s was not able to suppress *R. solanacearum* at day 14 after pathogen Akilainoculation.

In the pot experiments with the susceptible plant L390 (Fig. 5b), the population of R.s in the WF02 + R.s treatment was significantly decreased during the experimental period, except at 5 dpi. Conversely, the population of R.s in the B.s + R.s treatment was lower than that of the R.s control treatment only at 1 dpi.

Expression of tomato defense-related genes

PGPR can up-regulate defense-related enzymes in plants to increase plant defense capacity against disease. However, it is known that some pathogens produce effectors that are able to suppress plant immune responses (Hemetsberger et al. 2012; Jing et al. 2016). Accordingly, we sought to clarify whether pre-treatment with the inoculant still could induce the corresponding expression of plant defense-related genes after pathogen treatment. To elucidate the signaling pathways of PGPR-associated plant defenses, we determined the temporal expression patterns of the defense-related genes *PAL*, *PR1a*, *LOX*, and *ACO* in the leaves of the two tomato cultivars. Because the expression of most PGPR-related defense genes is induced at early stages after pathogen infection (Tan et al. 2013), we monitored changes within 3 dpi. In the B.s + R.s treatment with moderately resistant Micro-Tom, the relative expression levels of *PAL* and *PR1a* (SA signaling pathway) were induced by approximately three- and tenfold at 1 dpi, respectively (Fig. 6a, b). In the WF02 + R.s treatment with Micro-Tom, although there was no apparent elevation of *PAL* transcripts (Fig. 6a), *PR1a* gene expression was significantly increased by over 20-fold at 1 dpi (Fig. 6b). In contrast, the *LOX* and *ACO* genes (JA and ET signaling pathways, respectively) were not significantly activated (less than twofold) in Micro-Tom under either of the above treatment conditions (Fig. 6c, d).

In the experiments using the susceptible tomato L390, only the *LOX* gene was significantly induced (approximately tenfold) following B.s + R.s treatment at 0 and 2 dpi (Fig. 6c), whereas the other genes were expressed at very low levels. In the case of the WF02 + R.s treatment, *PAL* and *LOX* were elevated dramatically (approximately 7- and 40-fold, respectively) at 3 dpi, though *PR1a* and *ACO* levels were not significantly enhanced (Fig. 6b, d).

Discussion

Bacterial wilt is usually the most damaging crop disease in Taiwan, and a high incidence of this disease in tomato is caused by *R. solanacearum*. We selected the bacterial strain WF02 from nearly 40 isolates because it exhibited effective suppression of the tomato bacterial wilt pathogen in preliminary experiments (Huang et al., unpublished data). Strain WF02 was identified as *B. amyloliquefaciens* by molecular phylogenetic analyses and morphological tests (Figs. 1, 2). Several strains of this species have been successfully employed in the management of pests and diseases, including bacterial wilt (Chen et al. 2009a; Ryu et al. 2004; Yamamoto et al. 2014; Zhao et al. 2013).

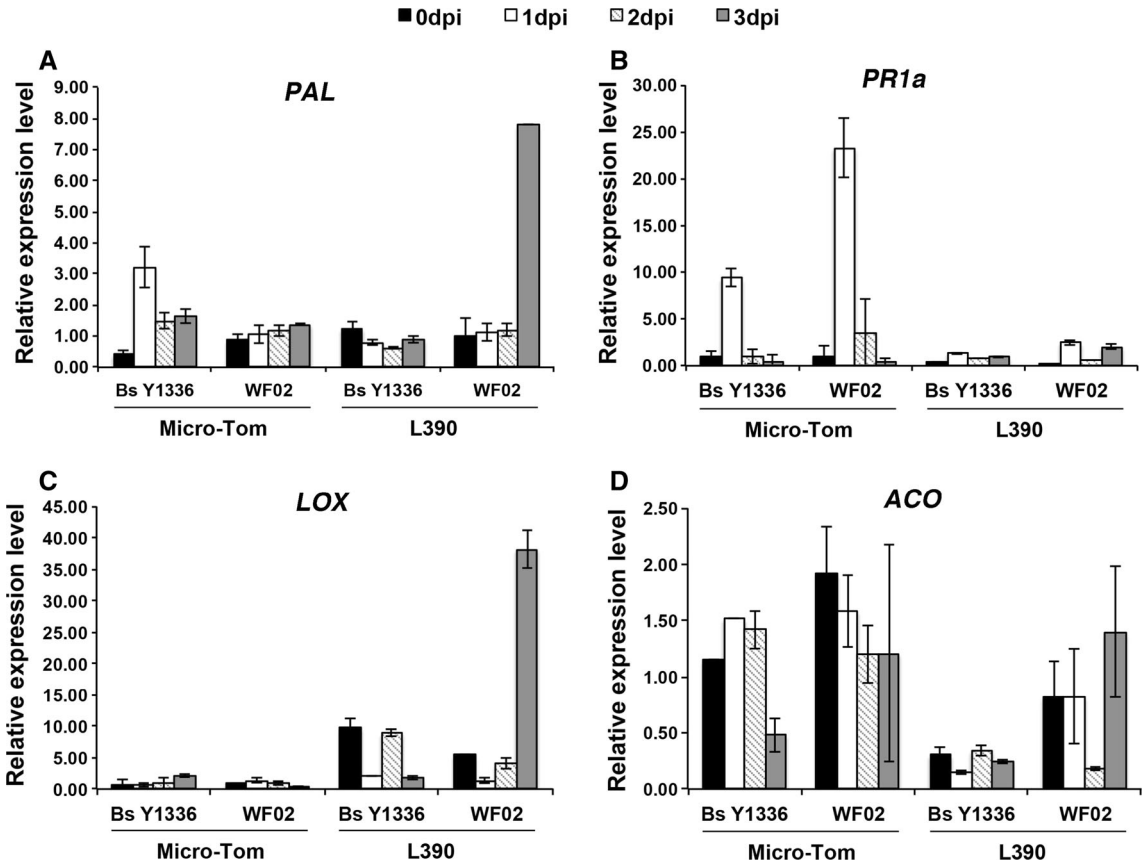

Fig. 6 Expression of defense-related genes in moderately resistant (Micro-Tom) and susceptible tomato cultivars (L390) over time. Total RNA was extracted from tomato leaves collected at 0–3 days post-inoculation (dpi) of the pathogen. The expression patterns of four defense-related genes (*PAL*, *PR1a*, *LOX*, and *ACO*) involved in the SA, JA and ET signaling pathways were analyzed for each treatment (B.s or WF02) by qPCR. The data are the mean ± SE of relative gene expression normalized to the control treatment (deionized/distilled water-treated plants, i.e., no inoculation with pathogen or inoculant) from three independent experiments

Biocontrol efficacy is largely influenced by the host plant genotype, and it is often difficult to obtain the same efficiency with a given biocontrol agent in different plant cultivars (Ryan et al. 2004). The commercial *B. subtilis* strain Y1336 (B.s) was used in this study, and this strain is reported to be capable of suppressing bacterial wilt on tomato plants (Yang et al. 2012). B.s could indeed reduce the mortality due to bacterial wilt in a moderately resistant tomato cultivar (Micro-Tom), it failed to suppress disease development in a susceptible cultivar (L390) (Fig. 4). In contrast, the WF02 isolate was able to suppress the disease severity to some extent in both cultivars more effectively than B.s. In Taiwan, most of the popular commercial tomato varieties valued for their flavor are susceptible cultivars, and only a few have moderate resistance against bacterial wilt (Chen et al. 2014). Accordingly, our results suggest that strain WF02 can serve as a potential biological agent to provide stable protection against *R. solanacearum* in agricultural applications.

In general, the biocontrol efficacy of PGPR against soil-borne pathogens is related to their antagonistic effects, root colonizing ability, and activation of host systemic resistance (Ji et al. 2008). As shown in Fig. 3, there was no significant difference between WF02 and B.s with regard to either in vitro antagonistic activity or siderophore production. It has been reported that biofilm formation promotes cell colonization efficiency and also enhances the local concentration of antibiotics surrounding the roots (Pieterse et al. 2014). We also evaluated the colonization ability of WF02 and B.s using the crystal violet staining method. As shown in Fig. 3c, both *Bacillus* strains exhibited a similar capacity to form biofilm in a 96-well plate, suggesting no significant difference in either the potential antagonistic effect or the colonizing ability between WF02 and B.s. Nonetheless, similar in vitro features between these two bacterial strains are not necessarily associated with their in vivo efficacy.

Plant immunity is usually accompanied by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling

(Choudhary and Johri 2009; Vanitha and Umesha 2011). SA accumulation is related to the systemic acquired resistance (SAR) induced by pathogen infection, and JA and ET are involved in induced systemic resistance (ISR) due to the effects of PGPR (van Loon et al. 1998). The expression of plant defense-related genes usually occurs quickly after PGPR application. We drenched the rhizosphere soil of tomato plants (Micro-Tom or L390) with the bacterial inoculant (B.s or WF02) and confirmed that not only JA/ET-related genes (LOX and ACO, respectively) but also an SA-related gene (PAL) were induced dramatically within 24 h in a preliminary test, (data not shown). This is consistent with a previous study using *Bacillus*-based biological control agents (Tan et al. 2013). In our study, we pre-treated with bacterial inoculant 7 days prior to plant pathogen infection, and we noticed that some plant immune-associated genes were still stimulated after treating with R.s. The SA-related genes *PAL* and *PR1a* were found to be activated by R.s treatment in both cultivars (Fig. S1), and their expression was enhanced in the presence of either B.s or WF02 (Fig. 6a, b, S1). These results suggest that soil application of either of these two *Bacillus* strains (B.s or WF02) can have additive or synergistic effects on plant systemic resistance to wilt disease in moderately resistant and susceptible tomato plants. Intriguingly, we observed extraordinarily high levels of *LOX* (JA) expression in both the moderately resistant tomato cultivar (Micro-Tom) under B.s + R.s treatment and the susceptible tomato cultivar (L390) under WF02 + R.s treatment. Because this signaling pathway does not operate independently, it remains to be investigated whether this remarkable increase in gene expression was due to different responses of the different plants or contributes to the prominent biological control activity observed.

Taken together, our results show that *B. amyloliquefaciens* strain WF02 can provide longer lasting protection against *R. solanacearum* than the commercial *B. subtilis* strain Y1336 and suppress the development of tomato wilt in either moderately resistant or susceptible tomato cultivars. This bacterium can secrete siderophore proteins to chelate ferric ions or synthesize antibiotics to suppress pathogen populations. The results of our in vitro antagonistic assay revealed the total production of antibacterial metabolites; therefore, the main antibiotic produced by WF02 needs to be studied in more detail to determine the relative role of the effective compound in the biocontrol activity of this strain. WF02 can protect plants from disease by inducing systemic immunity, including SAR and ISR. Furthermore, this bacterium is able to form endospores in response to harsh environments. These features indicate that strain WF02 has the potential to serve as an elite biological control agent for commercial purposes, and this should be verified in field trials.

Acknowledgments We appreciate Dr. Chiu-Ping Cheng (Graduate Institute of Plant Biology, National Taiwan University) and Dr. Jen-Chih Chen (Institute of Biotechnology, National Taiwan University) for sharing the bacterial and plant materials used in this study. We thank American Journal Experts for English language (Akila et al. 2011) editing. This study was supported by Grants from the Ministry of Science and Technology (101-2324-B-001-CC2 and 102-2313-B-002 -011 -MY3), National Taiwan University (102/103.SC 2B4 and 103R7602B429) and was also funded by Great Victory Chemical Industry Co. LTD.

References

Akila R, Rajendran L, Harish S, Saveetha K, Raguchander T, Samiyappan R (2011) Combined application of botanical formulations and biocontrol agents for the management of *Fusarium oxysporum* f. sp. *cubense* (Foc) causing *Fusarium* wilt in banana. Biol Control 57:175–183. doi:10.1016/j.biocontrol.2011.02.010

Almoneafy AA et al (2013) Synergistic effect of acetyl salicylic acid and DL-beta-aminobutyric acid on biocontrol efficacy of *Bacillus* strains against tomato bacterial wilt Tropical. Plant Pathol 38:102–113

Borneman J, Hartin RJ (2000) PCR primers that amplify fungal rRNA genes from environmental samples. Appl Environ Microbiol 66:4356–4360. doi:10.1128/Aem.66.10.4356-4360.2000

Buddenhagen I, Kelman A (1964) Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu Rev Phytopathol 2:203–230. doi:10.1146/annurev.py.02.090164.001223

Chen XH, Scholz R, Borriss M, Junge H, Mögel G, Kunz S, Borriss R (2009a) Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. J Biotechnol 140:38–44. doi:10.1016/j.jbiotec.2008.10.015

Chen YY, Lin YM, Chao TC, Wang JF, Liu AC, Ho FI, Cheng CP (2009b) Virus-induced gene silencing reveals the involvement of ethylene-, salicylic acid- and mitogen-activated protein kinase-related defense pathways in the resistance of tomato to bacterial wilt. Physiol Plant 136:324–335. doi:10.1111/J.1399-3054.2009.01226.X

Chen D, Liu X, Li C, Tian W, Shen Q, Shen B (2014) Isolation of *Bacillus amyloliquefaciens* S20 and its application in control of eggplant bacterial wilt. J Environ Manage 137C:120–127. doi:10.1016/j.jenvman.2014.01.043

Choudhary DK, Johri BN (2009) Interactions of *Bacillus* spp. and plants—with special reference to induced systemic resistance (ISR). Microbiol Res 164:493–513. doi:10.1016/j.micres.2008.08.007

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959. doi:10.1128/Aem.71.9.4951-4959.2005

Doornbos R, van Loon L, Bakker P (2012) Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A Rev Agron Sustain Dev 32:227–243. doi:10.1007/s13593-011-0028-y

Eden PA, Schmidt TM, Blakemore RP, Pace NR (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16s ribosomal-RNA-specific DNA. Int J Syst Bacteriol 41:324–325

Felsenstein J (1985) Confidence-limits on phylogenies—an approach using the bootstrap. Evolution 39:783–791. doi:10.2307/2408678

Fredslund L, Ekelund F, Jacobsen CS, Johnsen K (2001) Development and application of a most-probable-number-PCR assay to quantify flagellate populations in soil samples. Appl Environ Microbiol 67:1613–1618. doi:10.1128/Aem.67.4.1613-1618.2001

French E, De Lindo L (1982) Resistance to *Pseudomonas solanacearum* in potato: specificity and temperature sensitivity. Phytopathology 72:1408–1412. doi:10.1094/Phyto-72-1408

Ghareeb H, Bozso Z, Ott PG, Repenning C, Stahl F, Wydra K (2011) Transcriptome of silicon-induced resistance against *Ralstonia solanacearum* in the silicon non-accumulator tomato implicates priming effect. Physiol Mol Plant Pathol 75:83–89. doi:10.1016/J.Pmpp.2010.11.004

Gonzalez-Sanchez MA, de Vicente A, Perez-Garcia A, Perez-Jimenez R, Romero D, Cazorla FM (2013) Evaluation of the effectiveness of biocontrol bacteria against avocado white root rot occurring under commercial greenhouse plant production conditions. Biol Control 67:94–100. doi:10.1016/J.Biocontrol.2013.08.009

Granada GA, Sequeira L (1983) A new selective medium for *Pseudomonas-solanacearum*. Plant Dis 67:1084–1088. doi:10.1094/Pd-67-1084

Haas D, Defago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3:307–319. doi:10.1038/Nrmicro1129

Hall BG (2013) Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 30:1229–1235. doi:10.1093/molbev/mst012

Hemetsberger C, Herrberger C, Zechmann B, Hillmer M, Doehlemann G (2012) The Ustilago maydis effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity. PLoS Pathog 8:e1002684

Husen E (2003) Screening of soil bacteria for plant growth promoting activities in vitro. Indones J Agric Sci 4:27–31

Ji X, Lu G, Gai Y, Zheng C, Mu Z (2008) Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strain. FEMS Microbiol Ecol 65:565–573. doi:10.1111/j.1574-6941.2008.00543.x

Jing M, Guo B, Li H, Yang B, Wang H, Kong G, Zhao Y, Xu H, Wang Y, Ye W (2016) A *Phytophthora sojae* effector suppresses endoplasmic reticulum stress-mediated immunity by stabilizing plant binding immunoglobulin proteins. Nat Commun 7:11685. doi:10.1038/ncomms11685

Jogaiah S, Abdelrahman M, Tran LS, Shin-Ichi I (2013) Characterization of rhizosphere fungi that mediate resistance in tomato against bacterial wilt disease. J Exp Bot 64:3829–3842. doi:10.1093/jxb/ert212

Kelman A (1954) The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693–695

Kim DS, Cook RJ, Weller DM (1997) *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology 87:551–558. doi:10.1094/Phyto.1997.87.5.551

Kulkarni JH, Patil RB (1982) Production and utilization of extracellular slime by *Pseudomonas solanacearum* and its role on survival at different relative humidities. Acta Microbiol Pol 31:159–165

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556. doi:10.1146/annurev.micro.62.081307.162918

Marian MN et al (2012) MPN-PCR detection and antimicrobial resistance of *Listeria monocytogenes* isolated from raw and ready-to-eat foods in Malaysia. Food Control 28:309–314. doi:10.1016/j.foodcont.2012.05.030

Michel VV, Mew T (1998) Effect of a soil amendment on the survival of *Ralstonia solanacearum* in different soils. Phytopathology 88:300–305. doi:10.1094/Phyto.1998.88.4.300

Milling A, Babujee L, Allen C (2011) *Ralstonia solanacearum* extracellular polysaccharide is a specific elicitor of defense responses in wilt-resistant tomato plants. PLoS One 6:e15853. doi:10.1371/journal.pone.0015853

Munar-Vivas O, Morales-Osorio JG, Castañeda-Sánchez DA (2010) Use of field-integrated information in GIS-based maps to evaluate Moko disease (*Ralstonia solanacearum*) in banana growing farms in Colombia. Crop Prot 29:936–941. doi:10.1016/J.Cropro.2010.04.021

Nguyen MT, Ranamukhaarachchi SL (2010) Soil-borne antagonists for biological control of bacterial wilt disease caused by *Ralstonia solanacearum* in tomato and pepper. J Plant Pathol 92:395–405

Perez-Garcia A, Romero D, de Vicente A (2011) Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Curr Opin Biotechnol 22:187–193. doi:10.1016/J.Copbio.2010.12.003

Pieterse CM, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA (2014) Induced systemic resistance by beneficial microbes. Annu Rev Phytopathol 52:347–375. doi:10.1146/annurev-phyto-082712-102340

Raddadi N, Cherif A, Olizari H, Marzorati M, Brusetti L, Boudabous A, Daffonchio D (2007) *Bacillus thuringiensis* beyond insect biocontrol: plant growth promotion and biosafety of polyvalent strains. Ann Microbiol 57:481–494

Ramesh R, Joshi A, Ghanekar M (2009) Pseudomonads: major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia solanacearum* in the eggplant (*Solanum melongena* L.). World J Microbiol Biotechnol 25:47–55. doi:10.1007/s11274-008-9859-3

Ren JH, Li H, Wang YF, Ye JR, Yan AQ, Wu XQ (2013) Biocontrol potential of an endophytic *Bacillus pumilus* JK-SX001 against poplar canker. Biol Control 67:421–430. doi:10.1016/J.Biocontrol.2013.09.012

Ryan AD, Kinkel LL, Schottel JL (2004) Effect of pathogen isolate, potato cultivar, and antagonist strain on potato scab severity and biological control. Biocontrol Sci Technol 14:301–311. doi:10.1080/09583150410001665187

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW (2004) Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol 134:1017–1026. doi:10.1104/pp.103.026583

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. doi:10.1038/Nmeth.2089

Svercel M, Hamelin J, Duffy B, Moenne-Loccoz Y, Defago G (2010) Distribution of *Pseudomonas* populations harboring *phlD* or *hcnAB* biocontrol genes is related to depth in vineyard soils. Soil Biol Biochem 42:466–472. doi:10.1016/J.Soilbio.2009.11.030

Swanepoel AE (1992) Survival of South-African strains of biovar-2 and biovar-3 of *Pseudomonas solanacearum* in the roots and stems of weeds. Potato Res 35:329–332. doi:10.1007/Bf02357714

Swanson JK, Yao J, Tans-Kersten J, Allen C (2005) Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. Phytopathology 95:136–143. doi:10.1094/Phyto-95-0136

Tan S, Dong Y, Liao H, Huang J, Song S, Xu Y, Shen Q (2013) Antagonistic bacterium *Bacillus amyloliquefaciens* induces resistance and controls the bacterial wilt of tomato. Pest Manag Sci 69:1245–1252. doi:10.1002/ps.3491

Teitelbaum SL, Gammon MD, Britton JA, Neugut AI, Levin B, Stellman SD (2007) Reported residential pesticide use and breast cancer risk on Long Island. N Y Am J Epidemiol 165:643–651. doi:10.1093/aje/kwk046

Thomas HA (1942) Bacterial densities from fermentation tube tests. J Am Water Works Assoc 24:572–576

van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483. doi:10.1146/annurev.phyto.36.1.453

Vanitha S, Umesha S (2011) *Pseudomonas fluorescens* mediated systemic resistance in tomato is driven through an elevated synthesis of defense enzymes. Biol Plant 55:317–322. doi:10.1007/s10535-011-0045-3

Vanitha S, Niranjana S, Mortensen C, Umesha S (2009) Bacterial wilt of tomato in Karnataka and its management by *Pseudomonas fluorescens*. Biocontrol 54:685–695. doi:10.1007/s10526-009-9217-x

Wang JF, Olivier J, Thoquet P, Mangin B, Sauviac L, Grimsley NH (2000) Resistance of tomato line Hawaii7996 to *Ralstonia solanacearum* Pss4 in Taiwan is controlled mainly by a major strain-specific locus. Mol Plant Microbe Interact 13:6–13. doi:10.1094/Mpmi.2000.13.1.6

Wang LT, Lee FL, Tai CJ, Kasai H (2007) Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. Int J Syst Evol Microbiol 57:1846–1850. doi:10.1099/ijs.0.64685-0

Wu S, Peiffer M, Luthe DS, Felton GW (2012) ATP hydrolyzing salivary enzymes of caterpillars suppress plant defenses. PLoS One 7:e41947

Yamada S, Ohashi E, Agata N, Venkateswaran K (1999) Cloning and nucleotide sequence analysis of *gyrB* of *Bacillus cereus*, *B. thuringinesis*, *B. mycoides*, and *B-anthracis* and their application to the detection of *B. cereus* in rice. Appl Environ Microbiol 65:1483–1490

Yamamoto S, Shiraishi S, Kawagoe Y, Mochizuki M, Suzuki S (2014) Impact of *Bacillus amyloliquefaciens* S13-3 on control of bacterial wilt and powdery mildew in tomato. Pest Manag Sci. doi:10.1002/ps.3837

Yang W, Xu Q, Liu H, Wang Y, Wang Y, Yang H, Guo J (2012) Evaluation of biological control agents of *Ralstonia* wilt on ginger. Biol Control 62:144–151

Zhao QY, Ran W, Wang H, Li X, Shen QR, Shen SY, Xu YC (2013) Biocontrol of *Fusarium* wilt disease in muskmelon with *Bacillus subtilis* Y-IVI. Biocontrol 58:283–292. doi:10.1007/S10526-012-9496-5

Zhao X et al (2015) Collagen-like proteins (ClpA, ClpB, ClpC, and ClpD) are required for biofilm formation and adhesion to plant roots by *Bacillus amyloliquefaciens* FZB42. PLoS One 10:e0117414. doi:10.1371/journal.pone.0117414