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plant disease

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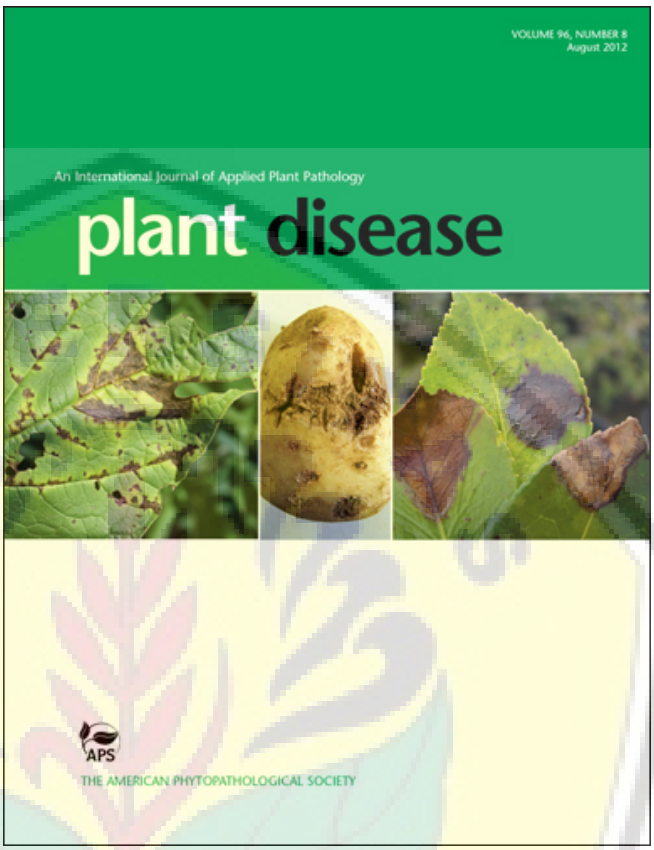
Symptoms of Xanthomonas leaf spot on sesame caused by *Xanthomonas* sp. (courtesy T. Isakeit et al., see page 1222); fissure scab lesion, a new symptom associated with potato common scab caused by a *Streptomyces* sp., on a potato tuber (courtesy R. Gouws and A. McLeod, see page 1223); leaf infected by a *Phytophthora* species (courtesy Y. Balci, see page 1080).



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Plant Disease

An International Journal of Applied Plant Pathology

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e-Xtra*

Utilization of Filamentous Phage ϕRSM3 to Control Bacterial Wilt Caused by *Ralstonia solanacearum*

Hardian S. Addy, Ahmed Askora, Takeru Kawasaki, Makoto Fujie, and **Takashi Yamada,** Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan

Abstract

Addy, H. S., Askora, A., Kawasaki, T., Fujie, M., and Yamada, T. 2012. Utilization of filamentous phage ϕRSM3 to control bacterial wilt caused by *Ralstonia solanacearum*. Plant Dis. 96:1204-1209.

The wide host range of *Ralstonia solanacearum*, causal agent of bacterial wilt, and its ability to survive for long periods in the environment restrict the effectiveness of cultural and chemical control measures. The use of phages for disease control is a fast-expanding trend of plant protection with great potential to replace chemical measures. The filamentous phage ϕRSM3 that infects *R. solanacearum* strains and inactivates virulence on plants is a potential agent for controlling bacterial wilt in tomato. We demonstrated that inoculation of ϕRSM3-infected cells into tomato plants did not cause bacterial wilt. Instead, ϕRSM3-infected cells enhanced the expression of pathogenesis-related (*PR*) genes, including *PR-1a, PR-2b*, and *PR7*, in tomato plants. Moreover, pretreatment with ϕRSM-infected cells protect tomato plants from infection by virulent *R. solanacearum* strains. The effective dose of ϕRSM3-infected cells for disease prevention was determined to be approximately 10^5 CFU/ml. Because the ϕRSM3-infected cells can grow and continue to produce infectious phage particles under appropriate conditions, ϕRSM phages may serve as an efficient tool to control bacterial wilt in crops.

Bacterial wilt, caused by *Ralstonia solanacearum*, is an important plant disease of many crops, damaging more than 200 species in 50 botanical families, occurring widely in the world, and persisting in the environment (24). Some management strategies are currently employed to control this disease such as chemical control, soil treatment, crop rotation, and resistant plants (14). Although soil treatments such as modification of soil pH or heat have occasionally been effective in suppressing the pathogen, they are limited to small-scale agriculture and are unfriendly to the environment (8). Crop rotation has not been effective, because *R. solanacearum* has a wide host range and survives for long periods in the soil (2). Although the use of resistant plant cultivars has been reported to be the most reliable method to control bacterial wilt, it is not completely effective because cultivars exhibit reduction in yield and plant quality, often lacking in stability or durability (7,24). Thus, alternative control methods for bacterial wilt, which are more effective, safer to applicators, and have lower environmental impact, are still needed.

Various studies have indicated that biological control of bacterial wilt could be achieved using antagonistic bacteria (10,27). Rhizobacteria like *Bacillus* spp. (31), *Pseudomonas* spp. (23), and *Streptomyces* spp. (17) are examples of bacteria with efficacy. Another potential biological agent to control bacterial wilt caused by *R. solanacearum* is avirulent mutants of *R. solanacearum* (15) through spontaneous or genetic mutation. Recently, we reported that filamentous ϕRSM-type phages (ϕRSM3 is a typical phage of this group) changed host bacterial cells to be avirulent after infection (5). Numerous studies reported that the use of avirulent strains could reduce disease severity. Avirulent strains of *Erwinia amylovora* and *E. chrysanthemi* reduced fire blight disease on apple (34) and soft rot disease on saintpaulia plants (*Saintpaulia ionantha*) (30), respectively. Ciardi et al. (11) reported that an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* increased the tolerance of tomato plants against bacterial spot disease. Similar effects were also demonstrated by avirulent strains of *R. solanacearum* against bacterial wilt in some plants (32,35).

Mechanisms underlying the control of bacterial diseases by avirulent strains are thought to involve production of bacteriocins and induction of plant resistance (4,9). Stem inoculation in tomato by an avirulent strain of the bacteria *Clavibacter michiganensis* subsp. *michiganensis* induced long-lasting, high-level-protection against the virulent bacterial strain (22). An avirulent strain of *Pseudomonas syringae* pv. *pisi* was shown to induce systemic acquired resistant (SAR) in pea (13). Moreover, Edreva (16) described a detailed mechanism by which an avirulent strain induces plant resistance via SAR through a salicylate acid signaling pathway.

The aim of this research was to demonstrate the application of filamentous phage ϕRSM to control bacterial wilt in tomato caused by *R. solanacearum*.

Materials and Methods

Bacterial strains and bacteriophage. *R. solanacearum* strain MAFF (The Ministry of Agriculture, Forestry, and Fisheries of Japan) 106603 (race 1, biovar 3, and phylotype I) was from the National Institute of Agrobiological Sciences (Japan). Avirulent strain M4S (race 1, biovar 3, and phylotype 1) was from the Leaf Tobacco Research Center, Japan Tobacco Inc. (33). The bacterial cells were cultured in CPG medium containing 0.1% casamino acids, 1% peptone, and 0.5% glucose at 28°C with shaking at 200 to 300 rpm (25). Strain MAFF 106603 carrying a green fluorescent protein (GFP)-expressing plasmid pRSS12 was described previously (26) and was cultivated in CPG containing kanamycin (50 µg/ml). In some cases, bacterial cells were cultivated in minimal medium (MM) containing 1.75 g of K_2HPO_4, 0.75 g of KH_2PO_4, 0.15 g of Na-citrate, 0.25 g of $MgSO_4$, and 1.25 g of $(NH_4)_2SO_4$ per liter (6). Bacteriophage ϕRSM3 (a member of filamentous ϕRSM-type phages belonging to the family *Inoviridae*) was described previously by Askora et al. (5). ϕRSM3 was routinely propagated using strain MAFF 106603 as the host. To collect sufficient phage particles, a total of 2 liters of bacterial culture was grown. When the cultures reached 0.1 unit at an optical density at 600 nm (OD_{600}), the phage was added at a dose of 0.01 to 0.05

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figures 1, 2, and 4 appear in color online.

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PFU/host cell. After further growth for 16 to 18 h, cells were removed by centrifugation in an R12A2 rotor in a Hitachi Himac CR21E centrifuge (Hitachi Koki Co. Ltd.), at 8,000 × *g* for 15 min at 4°C. The supernatant was passed through a 0.2-µm membrane filter and then phage particles were precipitated in the presence of 0.5 M NaCl and 5% polyethylene glycol 6000. Phage preparations were stored at 4°C until use. To isolate single colonies of MAFF 106603 infected with ϕRSM3, single ϕRSM3 plaques picked from assay plates covered with a MAFF 106603 lawn were streaked onto CPG plates. Single colonies were purified by repeated picking and streaking on CPG plates. The phage genomic DNA was isolated from cells in its replicative form and confirmed by restriction enzyme digestion.

In planta monitoring movement of bacterial cells. For real-time monitoring of *R. solanacearum* cells in planta, tomato seedlings were grown on agar medium and bacterial suspension was applied to the root apex as previously described (19,20). Briefly, seed of tomato (*Solanum lycopersicum* L) 'Oogata-fukuju' was obtained from Takii Co., Ltd.. For aseptic cultures, seed were surface sterilized with sodium hypochlorite containing 1 drop of Tween 20 and cultured in a square dish (sterile square Schale Number 2; Eiken Chemical Co., Ltd.) containing solid medium (0.15% Hyponex powder [Hyponex Japan Corp., Ltd.], 0.5% sucrose, and 1.5% agar, adjusted to pH 5.8) as described before (20). Plants were grown in a growth chamber (Sanyo Growth Cabinet) at 28°C under a photoperiod of 16 h of light and 8 h of darkness. During the culture period, the dishes in the chamber were tilted to a 45° angle to encourage roots to grow the surface of the medium. To inoculate into plants, bacterial cells of a GFP-expressing strain (ϕRSM3-infected or uninfected MAFF 106603 harboring pRSS12) (19,26) were cultured in CPG medium, and suspended in sterile distilled water at a density of 1×10^8 cells/ml. Tomato seedlings grown in culture dishes (9 days old) were cut at the tip of the taproot, 10 mm from the apex, with a razor blade; then, 1 µl of bacterial suspension was applied to the cut. After inoculation, the plants in the dishes were cultured in the growth chamber until observation. In each experiment, five seedlings were treated with ϕRSM3-infected cells and five with wild-type cells for control. Experiments were repeated three times. Bacterial cells in the plants were observed using an MZ16F fluorescence stereomicroscope (Leica Microsystem) equipped with GFP2 and GFP3 filters or an Olympus BH2 fluorescence microscope (Olympus). Microscopic images were recorded with a CCD camera (Keyence VB-6010).

Virulence assays. Cells of *R. solanacearum* were grown in CPG medium for 1 to 2 days at 28°C. After centrifugation, cells were resuspended in double-distilled (dd)H_2O at a density of 10^8 cells/ml (OD_{600} = 0.3). For the virulence assay, the bacterial cell suspension (1 µl) was injected with a needle into the major stem of tomato plants (4 to 6 weeks old with four leaves) at a site between the cotyledon and the first leaf. As a control, ddH_2O at the same density was injected in the same manner. Each bacterial strain was injected into five plants. Plants were cultivated in a Sanyo Growth Cabinet at 28°C (16 h of light and 8 h of darkness) for up to 1 week before evaluation of disease symptoms. The wilting symptoms were graded from 0 to 4 as described by Winstead and Kelman (37) and modified by Poueymiro et al. (29). To test the ability of ϕRSM3-infected cells to protect plants against subsequent infection by virulent cells, tomato seedlings were first injected with ϕRSM3-infected, MAFF 106603 cells, or the control as described above. The tomato plants were then injected with virulent cells

Table 1. List of primers used in quantitative reverse-transcriptase polymerase chain reaction

Primer name	Oligo sequences (5′→3′)	Target gene	Product (bp)
ACT-F	GTTGGACTCTGGTGATGGTGTTAG	*β-actin*	231
ACT-R	GTTTCAAGTTCCTGCTCGTAGTCA		
PR1a-F	TCCGAGAGGCCAAGCTATAACTAC	*PR1a*	165
PR1a-R	GCCTACAGGATCATAGTTGCAAGA		
PR2b-F	CAGGACAGATTTCACTTCCGTATG	*PR2b*	336
PR2b-R	AATGCTTCTCAAGCTCTGGATTCT		
PR69-F	CGAATTTGAAACACCTTATTGCAG	*PR7*	389
PR69-R	CGATTATGTCAGGTTTCAAGATGC		

Fig. 1. Effects of ϕRSM3 infection on *Ralstonia solanacearum* virulence. Tomato plants (4 weeks old) were injected with cells of MAFF 106603 uninfected or infected by ϕRSM3. As a control, plants were injected with double-distilled H_2O. Each bacterial strain was injected into 20 plants (3 displayed as examples). All plants injected with ϕRSM3-uninfected *R. solanacearum* cells showed wilting symptoms 1 week after injection. All plants injected with ϕRSM3-infected cells or control showed no wilting symptoms.

(MAFF 106603) at approximately 10 to 20 mm above the first injection point after various intervals (1 or 4 days, 1 or 2 weeks, or 1 or 2 months after the first injection). Wilting symptoms were evaluated as described above. Each bacterial strain was injected into 20 plants. The experiment was repeated three times.

Real-time quantitative reverse-transcription polymerase chain reaction. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed as described previously (3). First-strand cDNAs were synthesized from 1 µg of total RNA with a ReverTraAce reverse-transcriptase kit (Toyobo) and oligo dT primers according to the manufacturer's instructions. Specific gene primers were designed using Primer 3 (v. 0.4.0) software for real-time PCR. The negative control (to eliminate the possibility of residual DNA amplification) consisted of the same reaction except that the reverse transcriptase was omitted from the reaction mixture. Real-time qRT-PCR was performed with a SYBR premix Ex Taq kit (TakaraBio) using a LineGene fluorescence quantitative detection system (BioFlux). The 10 µl of reaction mixture contained 5 µl of SYBR premix Ex Taq, 1 µl of diluted cDNA, and 0.5 µM each gene primer (Table 1). PCR was performed under the following conditions: initial heating for 3 min at 95°C and 45 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 15 s. At the

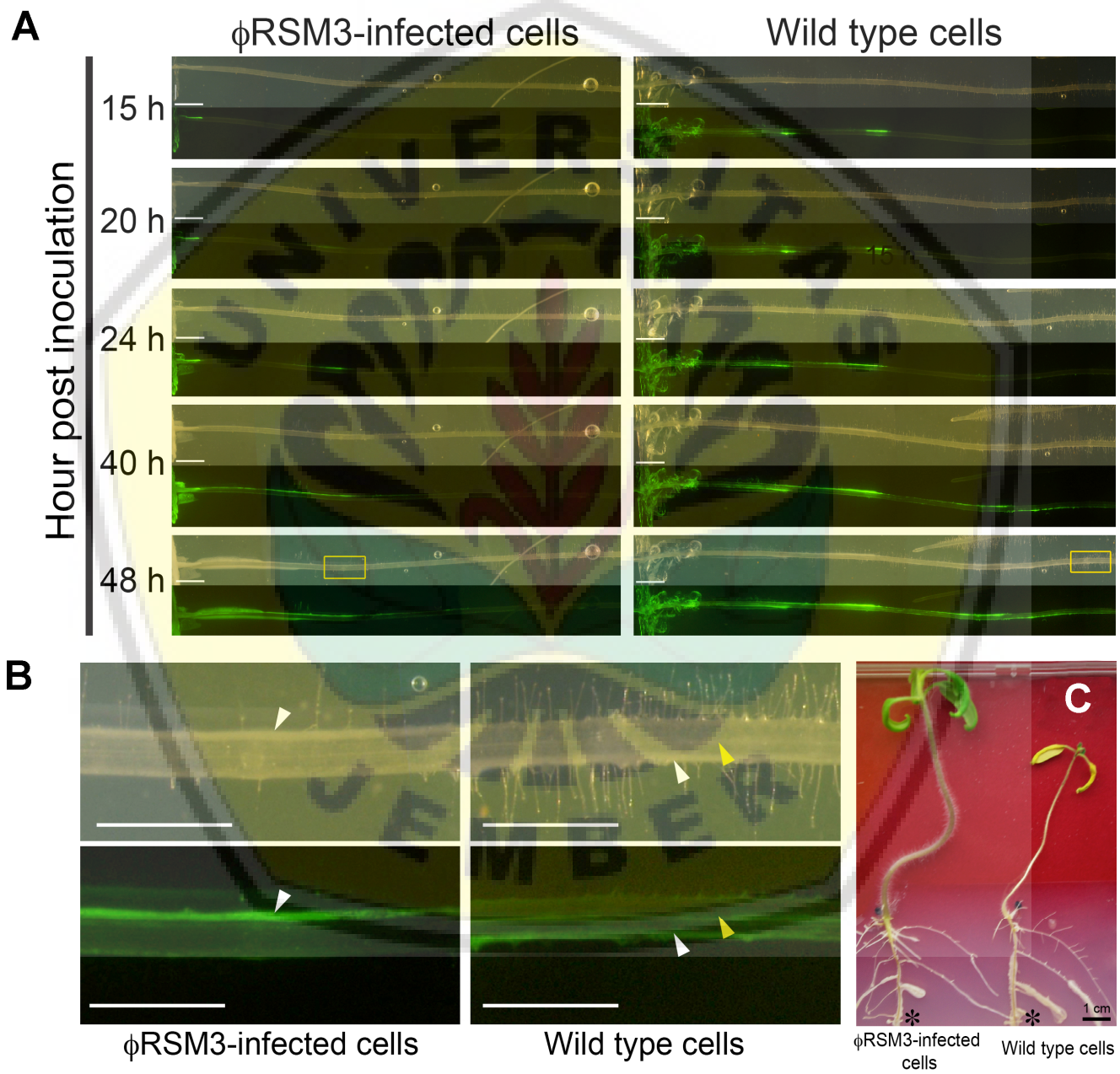

Fig. 2. In planta inhibition of *Ralstonia solanacearum* movement caused by ϕRSM3 infection. Tomato seedlings inoculated with **A,** noninfected bacterial cells (right) and ϕRSM3-infected cells (left) are compared. Green fluorescent protein (GFP) fluorescence from bacterial cells was observed at various times post inoculation (upper panel, bright-field image; lower panel, dark-field image). Bacterial penetration into the taproot, successive upward movement, and growth in the tissue was vigorous in the control seedling (inoculated with noninfected bacteria). Colonization of bacterial cells in the taproot was observed (yellow arrowhead). **B,** Comparison of the moving front of bacterial cells (local area boxed in A) at a higher magnification (upper panel, bright-field image; lower panel, dark-field image). Bacterial cells were observed to emerge from plant tissues of the seedling inoculated with uninfected cells. Bacterial growth and upward movement in the taproot were limited in seedling inoculated with ϕRSM3-infected cells and main GFP signal was due to the cells colonized the taproot surface (white arrowhead). **C,** Wilting symptom of tomato seedlings (10 days post inoculation). The control seedling inoculated with noninfected cells completely died, whereas the seedling with ϕRSM3-infected cells remained healthy without any wilting symptoms. White bars indicate 3 mm. Asterisk (*) refers to site of inoculation.

end of the program, the specificity of the primer set was confirmed by melting curve analysis (65 to 95°C with a heating rate of 0.5°C/min). Relative expression levels were calculated as the ratio of expression of each gene against the *β-actin* gene in tomato. Each experiment was repeated three times.

Results

Loss of virulence in ϕRSM3-infected *R. solanacearum*. In our previous work, Askora et al. (5) observed that ϕRSM-infected cells of *R. solanacearum* showed avirulent phenotypes. We confirmed this with cells at various densities. When a 1-µl cell suspension containing 10^5 (CFU) cells of MAFF 106603 was injected into the major stem of tomato plants (4 to 6 weeks old with four leaves), all plants showed wilting symptoms as early as 3 days post inoculation (dpi) and died 5 to 7 dpi (Fig. 1). In contrast, all 20 plants injected with ϕRSM3-infected MAFF 106603 cells showed no wilting symptoms until 4 weeks post inoculation. This was also the case with other host strains such as MAFF 106611; ϕRSM3-infected MAFF 106611 completely lost its virulence against tomato plants (*data not shown*). Bacterial growth and movements were monitored in real time under a fluorescent microscope. As indicated by GFP fluorescence, the bacterial cells accumulated in the xylem vessels and rapidly moved upward in tomato seedlings inoculated with noninfected bacteria (Fig. 2A). After inoculation, fluorescence intensity increased with time, indicating vigorous growth of bacterial cells. Meanwhile, most of the ϕRSM3-infected bacterial cells remained around the injection point (within 3 mm from the injection point), and their movement and growth were severely limited (Fig. 2A). Local taproot areas at the frontier of bacterial movement (Fig. 2A, box) were compared at a higher magnification in Figure 2B. In the seedling with ϕRSM3-infected bacterial cells, bacterial cells were limited around the inoculation point and the observed GFP signal was due to cells colonized outside the taproot, whereas bacterial cells multiplying and moving upward were eventually emerging from the xylem tissues and even covered the root surface. Seedlings inoculated with uninfected bacterial cells showed wilting symptoms 4 to 5 dpi and completely died 10 dpi, whereas seedlings with ϕRSM3-infected cells showed no wilting symptoms and remained healthy (Fig. 2C). Three repeated experiments showed statistically equivalent results.

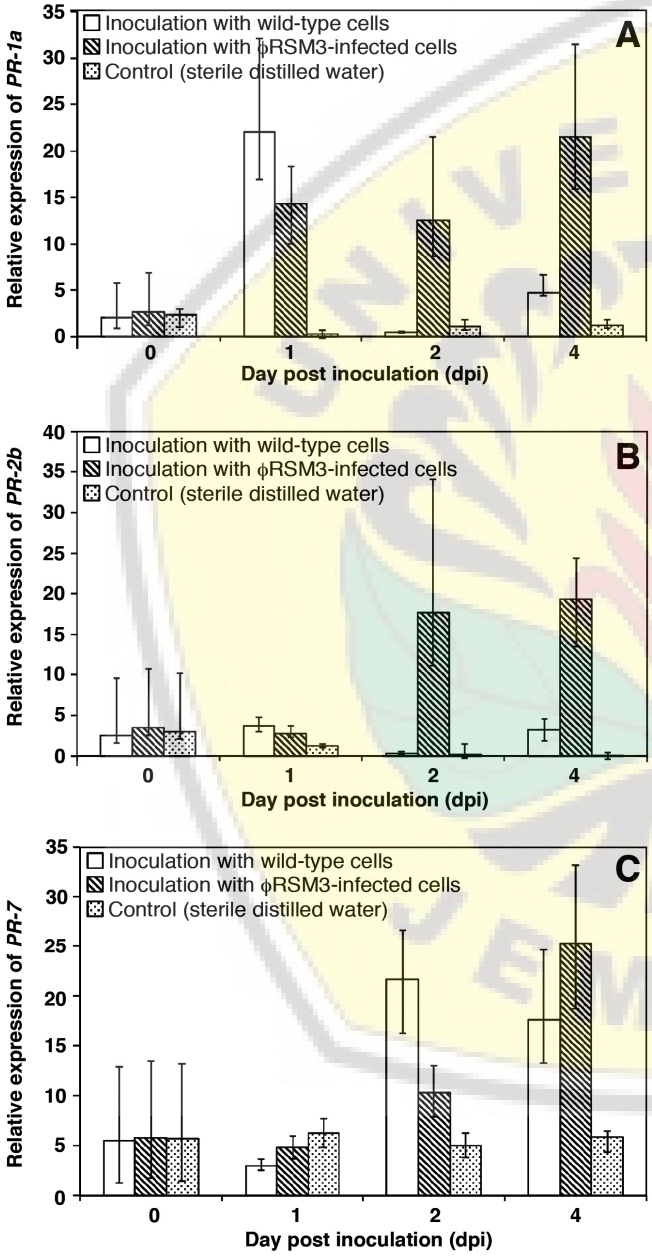

Fig. 3. Expression analysis of tomato genes involved in defense responses: pathogenesis-related (*PR*) genes **A,** *PR-1a*; **B,** *PR-2b*; and **C,** *PR-7*. Transcript levels of each gene were determined by quantitative reverse-transcription polymerase chain reaction from cDNAs isolated from leaves of tomato plants injected with ϕRSM3-infected bacterial cells (ϕRSM3 plant), with uninfected cells (no-phage plant), or with double-distilled H_2O (control). Expression value of each gene was normalized to that of the β-actin gene as an internal standard, and is shown as a relative values. Mean expression and standard deviation values were calculated from the results of three independent experiments.

Induction of pathogenesis-related genes in tomato by ϕRSM3-infected bacterial cells. The observation that ϕRSM3-infected bacterial cells were inhibited to grow and move in the inoculated tomato seedlings led us to examine the expression levels of some defense-related genes in the treated plants. After injection of bacterial cells (strain MAFF 106603), the tomato leaf just above the injection site was picked from each plant and subjected to the processes of RNA extraction and qRT-PCR. The expression of three pathogenesis-related (*PR*) genes, including those for *PR-1a* (an acidic *PR* gene), *PR-2b* (a basic *PR* gene), and *PR-7* (tomato P69) (36) was compared among plants with ϕRSM3-infected cells (ϕRSM3 plants), plants with uninfected cells (no-phage plants), and ddH_2O-injected plants (control). Data shown in Figure 3 indicated that the *PR-1a* gene was highly expressed in both no-phage plants and ϕRSM3 plants 1 dpi, and the expression level sharply decreased 2 dpi in no-phage plants. Meanwhile, the high expression levels continued until 4 dpi in ϕRSM3 plants (Fig. 3A). In the case of *PR-2b*, its expression was very low in no-phage plants until 4 dpi whereas significant levels of expression were detected after 2 dpi in ϕRSM3 plants (Fig. 3B). The expression of the gene for *PR-7* was induced 2 dpi and continued until 4 dpi in both ϕRSM3 plants and no-phage plants. However, *PR-7* expression started more slowly in response to phage-infected cells but its levels were higher in ϕRSM3 plants than in no-phage plants (Fig. 3C). In control plants, the expression of these three genes was consistently low. In ϕRSM3 plants, the expression of the defense genes was stably activated and maintained at high levels, likely due to reduced activity of virulence genes, including *hrpB,* responsible for type III effector secretion in ϕRSM3-infected bacterial cells. In all three repeated experiments, similar results were observed.

Pretreatment of tomato seedlings with ϕRSM3-infected *R. solanacearum* cells protected plants from subsequent infection by virulent cells. In addition to their loss of virulence, ϕRSM3-infected cells protected tomato plants against subsequent challenge with virulent *R. solanacearum* cells. Tomato plants were inoculated with ϕRSM3-infected MAFF 106603 cells as described above. Then, 1 day later, plants were injected with noninfected virulent bacteria at approximately 10 to 20 mm above the first injection point. No wilting symptoms were observed in ϕRSM3-treated plants for at least 4 weeks after inoculation (Fig. 4), whereas control plants without ϕRSM3-infected cells or those that had been pretreated with *Escherichia coli* cells all died within 1 week. Under standard virulence assay conditions (see Materials

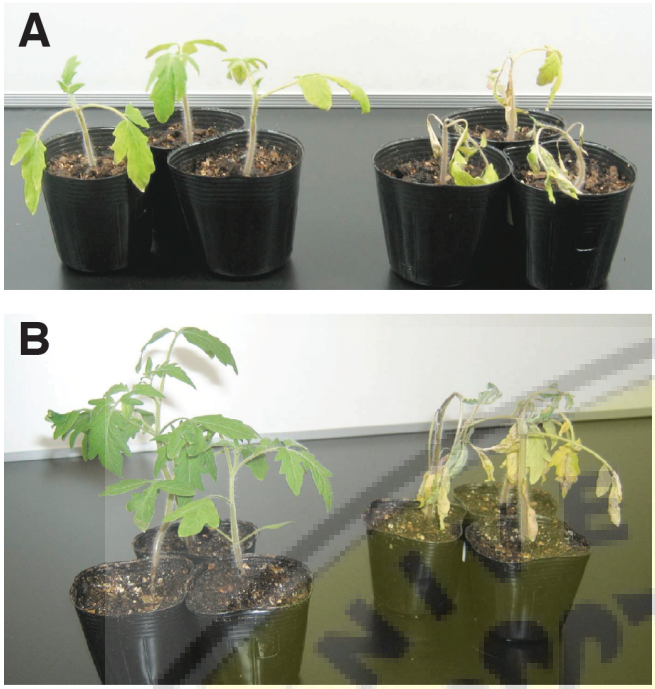

Fig. 4. Enhanced resistance against subsequent infection by virulent cells in tomato seedlings pretreated with ϕRSM3-infected cells. Tomato seedlings (10 days old) were first injected with ϕRSM3-infected cells (left) or *Escherichia coli* JM109 (right). Tomato plants were then injected with virulent cells (MAFF 106603) after various intervals (**A,** 1 day or **B,** 2 weeks). All plants pretreated with ϕRSM3-infected cells grew normally and showed no wilting symptoms. All control plants showed wilting symptoms and died within 1 week after the second injection.

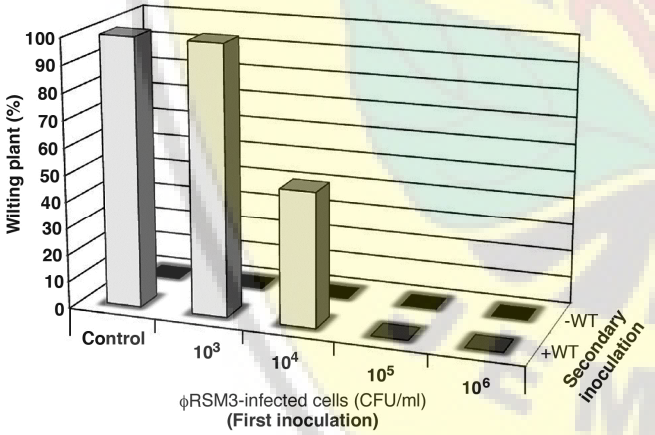

Fig. 5. Dose effects of ϕRSM3-infected cells for protecting plants against second inoculation of virulent cells. One week after the first injection with ϕRSM3-infected cells at different doses, the tomato plants were inoculated with virulent bacterial cells. Wilting symptoms were evaluated as described above. Twenty plants were subjected to each assay.

and Methods), effective doses of ϕRSM3-infected cells were determined. ϕRSM3-infected cells at a density lower than 10^3 CFU/ml showed no prevention effects, 50% protection was observed at 10^4 CFU/ml, and all plants survived with cell density greater than 10^5 CFU/ml (Fig. 5). This wilt-prevention effect was observed in plants as young as 1 week old (*data not shown*). Two months after the treatment with ϕRSM3-infected cells, inoculation with virulent cells could not cause wilting symptoms (Table 2). Therefore, once plants were treated with ϕRSM3-infected cells, the prevention effect lasted for up to 2 months (Table 2). Similar protective effects against infection by MAFF 106603 were also achieved by treating tomato plants with ϕRSM3-infected MAFF 106611 or M4S (a spontaneously occurring avirulent strain) (28) as well as MAFF 211271 (race 3) and MAFF 211272 (race 4) (*data not shown*).

Discussion

Like other filamentous Ff-type phages (*Inoviridae*), infection with ϕRSM3 does not cause host bacterial cell lysis. Instead, it establishes a persistent association between the host bacteria and phage, releasing phage particles from the growing host bacterial cells. Therefore, infection by phage can mediate conversion of the host bacterial phenotype. Upon infection by ϕRSM3, host cells demonstrate complete loss of virulence. The loss of virulence in host bacterial cells is caused by repression of some host genes, including *phcA* and *hrpB*, by phage-encoded open reading frame 15 (1). Furthermore, once inoculated with ϕRSM3-infected *R. solanacearum* cells, plants showed stable resistance upon inoculation with virulent bacteria. This resistance was induced as early as 1 dpi and lasted for up to 2 months. After inoculation of bacterial cells into tomato plants, *PR* genes (*PR-1a*, *PR-2b*, and *PR-7*) were highly expressed in ϕRSM3 plants, especially at 4 dpi. In the case of *PR-1a*, the expression level was high in both no-phage plants and ϕRSM3 plants as early as 1 dpi but it sharply decreased 2 dpi in no-phage plants. This blockage of host response by wild-type (no-phage) bacterial cells may be caused through the type III secretion system (T3SS) that allows the translocation of effector proteins into plant cells (21). Contrasting to this, the high expression levels of *PR-1a* continued until 4 dpi in ϕRSM3 plants, suggesting reduced activity of T3SS in ϕRSM3-infected bacterial cells. Actually, the expression of *hrpB*, the regulator gene of T3SS-encoding and effector genes (12), was reduced in ϕRSM3-infected bacterial cells (1). The expression of *PR-7* started more slowly in response to phage-infected cells but its levels became much higher in ϕRSM3 plants than in no-phage plants at 4 dpi. This looks like a general resistance response (36) overlaid with a specific response to the ϕRSM3-infected *R. solanacearum*. This specific response was also seen in the expression of *PR-2b*, which was very low in non-phage plants until 4 dpi.

Because the ϕRSM-infected cells can grow and continue to produce infectious phage particles under appropriate conditions, ϕRSM phages may serve as an efficient tool to control bacterial wilt in crops by decreasing the virulence of the pathogen. The use of avirulent mutants of *R. solanacearum* for bacterial wilt biocontrol has already been reported (32,35), and the protective nature of such pre-interactions on tomato plants is established. Compared with avirulent mutants, advantages of the ϕRSM3-infected cells can be explained as follows. ϕRSM phages with compensating host ranges infect a wide range of strains of different races. Askora et

Table 2. Prevention of the second infection through ϕRSM3-pretreatment in tomato

	Interval between the first and second injections[a]					
Strains	**1 day**	**4 days**	**1 week**	**2weeks**	**1 month**	**2 months**
MAFF 106603 (ϕRSM3)	–	–	–	–	–	–
MAFF 106611 (ϕRSM3)	–	–	–	–	–	–
M4S	–	–	–	–	–	–
Escherichia coli JM109	+	+	+	+	+	+

[a] Symbols: + = wilting 1 week after injection with the second virulent cells (MAFF 106603) and – = no wilting 1 week after injection with the second virulent cells (MAFF 106603).

al. (5) demonstrated that ϕRSM1, a ϕRSM3-related phage, has a host range different from that of ϕRSM3. All tested strains of *R. solanacearum* of different races or biovars were susceptible to one of the two phages (5). It is likely that a mixture of ϕRSM1 and ϕRSM3 would be particularly effective against field-isolated, uncharacterized *R. solanacearum* strains. Moreover, infection with ϕRSM phage is very easy and reliable. There is no need of mutagen treatment or transposon introduction and following selection and characterization before use. After inoculation into plants, ϕRSM phage released from infected cells can infect new susceptible cells, so that the protection effects can be long lasting. This contrasts with the case of biocontrol with avirulent mutants, where co-inoculated or second-inoculated virulent cells colonized the stem tissue at a high density (18).

For further development of this method, two ways can be proposed. First, a strain of interest just isolated from a wilted plant on site can be converted to avirulent by infection with ϕRSM phage for biocontrol purpose. This avirulent strain may be inoculated into healthy plants growing around the wilting spot to prevent further infection and expansion of the disease. Second, crop seedlings or young plants grown in nursery pots can be treated with ϕRSM-infected *R. solanacearum* cells before planting to fields for prevention of bacterial wilt, because the protection effects will be long lasting (for more than 2 months), as demonstrated above. The use of ϕRSM-infected cells for biocontrol of *R. solanacearum* can be used in combination with another method using lytic phages such as ϕRSL1 (20,38), which is most effective for wilt prevention when plant seedlings are treated.

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