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Monitoring of Phytopathogenic *Ralstonia solanacearum* Cells Using Green Fluorescent Protein-Expressing Plasmid Derived from Bacteriophage ϕRSS1

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A green fluorescent protein (GFP)-expressing plasmid was constructed from a filamentous bacteriophage ϕRSS1 that infects the phytopathogen *Ralstonia solanacearum*. This plasmid designated as pRSS12 (4.7 kbp in size) consists of an approximately 2248 bp region of the ϕRSS1 RF DNA, including ORF1-ORF3 and the intergenic region (IG), and a Km cassette in addition to the GFP gene. It was easily introduced by electroporation and stably maintained even without selective pressure in strains of *R. solanacearum* of different races and biovars. Strong green fluorescence emitted from pRSS12-transformed bacterial cells was easily monitored in tomato tissues (stem, petiole, and root) after infection as well as from soil samples. These results suggest that pRSS12 can serve as an easy-to-use GFP-tagging tool for any given strain of *R. solanacearum* in cytological as well as field studies.

[**Key words:** bacterial monitoring, green fluorescent protein (GFP), phage-derived plasmid, bacterial wilt, *Ralstonia solanacearum*]

Ralstonia solanacearum is a soil-borne gram-negative bacterium known to be the causative agent of bacterial wilt in many important crops (1, 2). This bacterium has an unusually wide host range with over 200 species belonging to more than 50 botanical families (1). It infects roots and exhibits strong tissue-specific tropism within the host, specifically invading and extensively multiplying in the xylem vessels. In the field, *R. solanacearum* is easily spread via contaminated irrigation water and can survive for many years in association with alternate hosts. In cropping fields, gardens, or greenhouses, once identified as being infected, plants must be destroyed and soil and water draining systems that could potentially be contaminated with the bacterium must be treated with chemical bacteriocides such as quaternary ammonia compounds, peroxygen compounds, or bleaches. Because of the limited efficiency of current integrated management strategies, bacterial wilt continues to be an economically serious problem for field-grown crops in many subtropical, tropical, and warm areas of the world (1, 3).

Recently, Yamada *et al.* (4) isolated and characterized various types of bacteriophage that specifically infect *R. solanacearum* strains. These phages may be useful as a tool for molecular biological studies of *R. solanacearum* pathogenicity. They could also be used for the specific and efficient detection and control of harmful pathogens in cropping ecosystems as well as growing crops. One of them, ϕRSS1, was characterized as an Ff-like phage (Inovirus) on the basis of its filamentous morphology, genomic ssDNA, and infection cycle. The genome of ϕRSS1 is 6662 nt long with a GC content of 62.6%, which is comparable to that of *R. solanacearum* GMI1000 (66.97%; 5). There are 11 open reading frames (ORFs) located on the same strand (6). In general, the genome of Ff-like phages is organized in a modular structure, in which functionally related genes are grouped (7). Three functional modules are always present. The replication module contains the genes encoding rolling-circle DNA replication and single-strand DNA (ssDNA) binding proteins, *gII*, *gV*, and *gX* (8). The structural module contains genes for the major (*gVIII*) and minor coat proteins (*gIII*, *gVI*, *gVII*, and *gIX*), and gene *gIII* encodes the host recognition or adsorption protein pIII (9). The assembly and secretion module contains the genes (*gI*, and *gIV*) for morphogenesis and extrusion of the phage particles (10). Gene *gIV* encodes protein pIV, an aqueous channel (secretin) in the outer membrane through which phage particles exit from the host cells. The ϕRSS1 genes fit well with the general arrangement of Ff-like phages. A survey of the databases for amino acid sequences of ϕRSS1 ORFs revealed significant homology to the Ff-like phage proteins such as ORF2 (pII homologue), ORF4 (pVIII homologue), ORF7 (pIII homologue), ORF8 (pVI homologue) and ORF9 (pI homologue). To verify the replication origin, an autonomously replicating plasmid was derived from ϕRSS1 DNA. A one-third portion (approximately 2250 b) of ϕRSS1 DNA that lacks the modules for structural proteins and morphogenesis (ORF4-

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ORF11) was connected to a Km cassette and the resulting plasmid of approximately 3.7 kbp in size (pRSS11) was stably maintained in *R. solanacearum* cells (6).

In this study, pRSS11 was further modified to express green fluorescence protein for the monitoring of *R. solanacearum* cells in wiliting plants and cropping fields.

MATERIALS AND METHODS

Bacterial strains and culture conditions *R. solanacearum* strains were obtained from the following culture collections: strain M4S from the Leaf Tobacco Research Center, Japan Tobacco (JT), and strains MAFF106611, MAFF211270, MAFF211272, and MAFF301558 from the National Institute of Agrobiological Sciences, Japan. The bacterial cells were cultured in CPG medium containing 0.1% casamino acids, 1% peptone and 0.5% glucose (11) at 28°C with shaking at 200–300 rpm.

DNA manipulations Standard molecular biological techniques for DNA isolation, digestion with restriction enzymes and other nucleases, and construction of recombinant DNAs were followed according to Sambrook and Russell (12). Phage DNA was isolated from purified phage particles by phenol extraction. In some cases, extrachromosomal DNA was isolated from phage-infected *R. solanacearum* cells by the mini-preparation method (13). The autonomously replicating plasmid pRSS11 constructed in our previous experiment (6) contains 2248 bases of ϕRSS1 DNA lacking the modules for structural proteins and morphogenesis (ORF4-ORF11), and the Km cassette excised from plasmid pUC4-KIXX (Amersham Biosciences, Piscataway, NJ, USA). The same ϕRSS1 DNA fragment was ligated to a 2.5 kbp fragment containing the Km cassette and the gene for GFP excised from pGFPuv-Km by blunt-end ligation, resulting in pRSS12 (Fig. 1). pGFPuv-Km was formed by inserting the Km cassette from pUC4-KIXX into the *Eco*RI site of pGFPuv (Takara Bio, Kyoto). The orientation of each gene was confirmed by restriction enzyme mapping. PCR primers F1 and R1 were the same as previously described (6) and F2 and R2 were as follows: F2, 5′-GAGCGCCGAATTCGCAAA CCGCCTCTCC, and R2, 5′-TTGACACCAGACAAGTTGGTAA TGGTAG.

The plasmid pRSS12 was introduced into the cells of various *R. solanacearum* strains by electroporation using a Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, USA) with a 2 mm cell at 2.5 kV according to the manufacturer's instructions. Transformants were selected on CPG plates containing 50 μg/ml kanamycin (Meiji Seika, Tokyo).

Stability and curing of pRSS12 in *R. solanacearum* cells pRSS12-transformed MAFF106611 cells were cultured in CPG without kanamycin. Aliquots of the culture were taken at appropriate intervals for colony formation assay. The ratio of colony number on Km-containing plates to that on Km-minus plates (without selective pressure) was calculated to evaluate the plasmid stability. For the control, assays using pKZ27-transformed (14) MAFF106611 cells were carried out in parallel. Enforced curing of pRSS12 from transformed cells was performed by EtBr treatment. PRSS12-transformed MAFF106611 cells were incubated in CPG with different EtBr concentrations (0, 3, and 10 μg/ml). At appropriate intervals, samples were taken for colony formation assay as described above.

***In planta* detection of *R. solanacearum* cells** *R. solanacearum* cells were grown in CPG medium for 1–2 d at 28°C. GFP fluorescence of pRSS12-transformed MAFF106611 cells was observed under an Olympus BH2 fluorescence microscope (Olympus Corp., Tokyo) with a GFP filter. For inoculation to plants, the bacterial cells were suspended in distilled water at a density of 10^8 cells/ml.

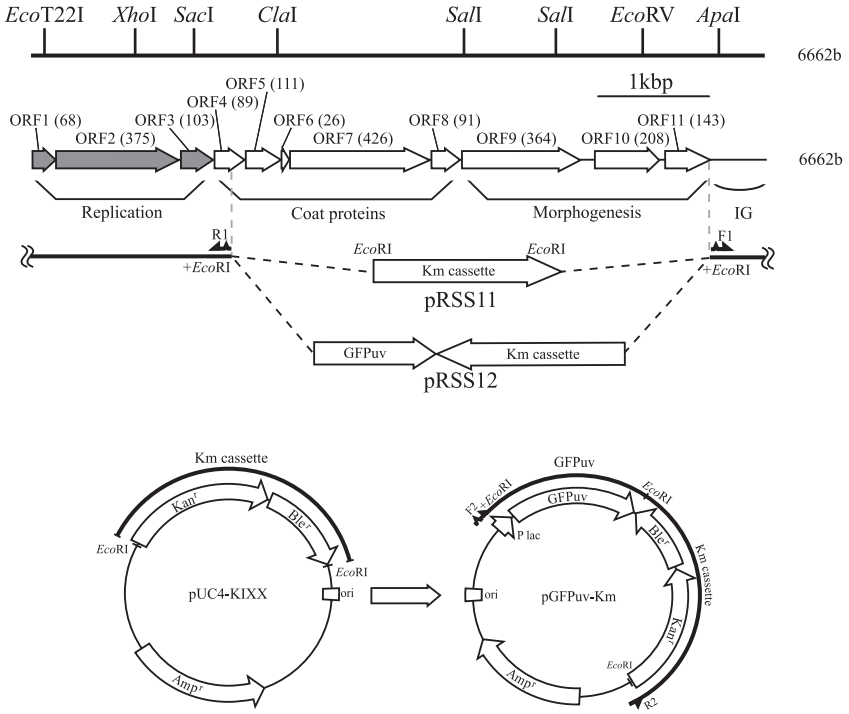

FIG. 1. Construction of pRSS12 from bacteriophage ϕRSS1 DNA. A portion containing ORF1-ORF3 and the intergenic region (IG) of ϕRSS1 DNA was amplified by PCR. This fragment was connected to a 1.5 kbp Km-cassette cut out from pUC4-KIXX with *Eco*RI, resulting in pRSS11 (6). The same ϕRSS1 fragment was connected to a 2.5 kbp fragment containing the Km cassette and the gene for GFP cut out from pGFPuv-Km, resulting in pRSS12. pGFPuv-Km was formed by inserting the Km cassette from pUC4-KIXX into the *Eco*RI site of pGFPuv. The orientation of each gene was confirmed by restriction enzyme mapping. F1, F2, R1, and R2 indicate primers for PCR. For details of ϕRSS1 ORFs, see Kawasaki *et al.* (6). Circular plasmids pRSS11 and pRSS12 are shown in linearized forms.

The growth and movement of pRSS12-transformed cells in leaf and root tissues were examined after inoculation of the bacterial suspension to a tomato leaf and root cuts. To observe the accumulation of the bacterial cells in the xylem vessel of wilting plants, the cell suspension was injected with a needle into the major stem of tomato plants (4 weeks old with 4–6 leaves) at a site 1 cm above the soil level (just above the cotyledons). Plants were cultivated in a Sanyo Growth Cabinet (Sanyo, Osaka) at 28°C (16 h light/8 h dark) for 1–4 weeks before detailed examination. Symptoms of wilting were graded from 1 to 5 as described by Winstead and Kelman (15). Plant tissues were observed using a Leica MZ16F stereomicroscope with GFP2 and three filters (Leica Microsystems, Heidelberg, Germany).

Recovery of *R. solanacearum* cells from soil samples One milliliter culture of pRSS12-transformed MAFF106611 cells (~10^9 cells/ml) was added to each soil sample (1.0 g wet weight): sample A, a natural field soil (pH 6.8); sample B, an artificial soil (mixture of 32% kaolinite, 38% vermiculite and 30% peat moss). After mixing by vortex, the soil samples were allowed to stand at room temperature for 1 h before centrifugation at 600 rpm (Tomy LX-120 centrifuge with a 3815-10P rotor) at 4°C. After appropriate dilution with distilled water, the fluorescence intensity was measured using an FP-6500 spectrofluorometer (Jasco, Essex, United Kingdom) at 395 nm excitation and 509 nm emission wavelengths.

RESULTS AND DISCUSSION

Construction of a GFP-expressing plasmid from ϕRSS1 DNA In our previous work, a minireplicon or plasmid was derived from ϕRSS1 (6). The plasmid designated as pRSS11 (3.7 kbp in size) consists of a 2248 nt portion including ORF1-ORF3 and IG of ϕRSS1 DNA and a Km cassette (Fig. 1). pRSS11 was stably maintained in cells of *R. solanacearum*, indicating that the replication origin and proteins required for the replication are confined within this ϕRSS1 DNA fragment. This fragment was ligated to an approximately 2.5 kbp fragment containing the gene for GFP and the Km cassette which was cut out from pGFPuv-Km (Fig. 1). The resulting plasmid (pRSS12) of approximately 4.7 kbp in size transformed the cells of various *R. solanacearum* strains into Km-resistant (50 μg/ml) at frequencies of approximately 10^7 cfu/μg DNA. All the colonies that appeared on selection plates (CPG containing 50 μg/ml kanamycin) emitted strong green fluorescence under UV irradiation (Fig. 2A) and all the bacterial cells observed by fluorescence microscopy showed strong green fluorescence (Fig. 2B). Fluorescence emission of GFP from pRSS12-transformed cells was determined to be 130.6 relative units/10^7 cells. The transformation efficiency was almost the same for strains of different races or biovars, including M4S (race 1, biovar 3) MAFF106611 (race 1, biovar 4), MAFF211270 (race 1, biovar N2), MAFF211272 (race 4, biovar 4), and MAFF301558 (race 3, biovar N2). The transformed cells (10^{10} cells/ml) contained approximately 50 ng/ml pRSS12 DNA, corresponding to a single copy per cell. pRSS12 was very stably maintained in *R. solanacearum* strains; for example, pRSS12-transformed MAFF106611 cells almost perfectly retained the plasmid after cultivation for 100 generations (12 d) without selective pressure (in CPG without kanamycin) (Fig. 3A). This stability is comparable to that of pRSS11, also derived from ϕRSS1 DNA (6). For comparison, plasmid pKZ27 (an incQ plasmid derived from pKZ240, 14) was easily lost from MAFF106611 cells under the same incubation condition; after two days, more than 90% of the cells lost the plasmid (Fig. 3A). This remarkable stability of pRSS12 as well as pRSS11 may be attributed to the characteristic replication mechanism of filamentous phage genomes. In general, filamentous phage DNA replication occurs in two steps (16, 17). First, an RNA-primed minus strand is made on the genomic plus strand. Then, a new plus strand is made on the resulting dsDNA. The replication of the plus strand proceeds via a rolling-circle mechanism on the replicative dsDNA template. The phage replication initiation protein, pII, plays major roles in this process. It introduces a single-strand break on a specific site on the plus strand of the negatively supercoiled RF (RFI). The 3′-end of the nick serves as the primer for plus strand synthesis, which proceeds with a concomitant displacement of the old plus strand in a single-stranded form. Upon completion of a round of replication, pII functions in termination by cleaving and circularizing the displaced single-stranded genomic DNA. In the case of coliphage fd, fd-miniplasmids required only the pII gene (*gII*) and the replication origin within IG for their stable replication independent of the *polA* function of the host cell (18). Both pRSS11 and pRSS12 contained the ϕRSS1 pII (ORF2) and IG. The stability of pRSS12 is also consistent with other examples of autonomous replicating plasmids derived from Ff-like phages. For example, a 2028-bp fragment of *Xanthomonas campestris* pv. *campestris* phage ϕLF was maintained autonomously as a minireplicon (pOR1) under nonselective conditions (19). This fragment covers regions encompassing *gII* to *gVIII* and IG between *gXI* and *gII*. This stability of pRSS12 in transformed cells is very important and useful during the experimental monitoring of bacterial cells in plant tissues or cropping fields where no selective pressure can be applied.

Meanwhile, pRSS12 can be cured by treatment with EtBr under standard conditions (20). When pRSS12-transformed cells were incubated in CPG containing EtBr (3 μg/ml), approximately 50% of the cells lost the plasmid after 3 d. The plasmid curing was more efficient with 10 μg/ml EtBr: after 3-d of incubation, most of the cells lost the plasmid (Fig. 3B). The plasmid curing can be used to rule out the spontaneous integration of the plasmid in the bacterial genome.

Monitoring of *R. solanacearum* cells in infected plants by GFP fluorescence Compared with the wild-type cells of *R. solanacearum* strain MAFF106611, pRSS12-transformed cells showed no significant differences in physiological properties including the growth rate, formation of extracellular polysaccharides (EPS), pigmentation (other than GFP), colony fluidity, virulence traits, and host specificity (data not shown). Green fluorescence emitted from pRSS12-transformed MAFF106611 cells could be used as a marker to monitor the behaviors of the bacterial cells during infection establishment and wilt disease progress. To demonstrate this, the bacterial cell suspension (1.0 OD_{600} unit corresponding to 2×10^9 cells/ml) was applied to a cut on a tomato leaf and then the cells were observed by GFP fluorescence. The GFP fluorescence intensity strongly increased 12 h after inoculation and the cells were observed to invade into the leaf tissues along the xylem vessels (Fig. 4A). Ac-

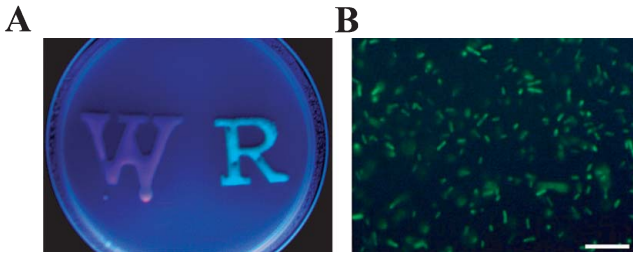

FIG. 2. Green fluorescence emitted from pRSS12-transformed colonies (A) and cells (B) of *R. solanacearum* MAFF106611. (A) W and R, colonies of nontransformed and pRSS12-transformed MAFF106611 cells on CPG plate observed under UV light (at 365 nm), respectively. (B) Fluorescence microscopy observation of transformed cells. Bar represents 5 μm.

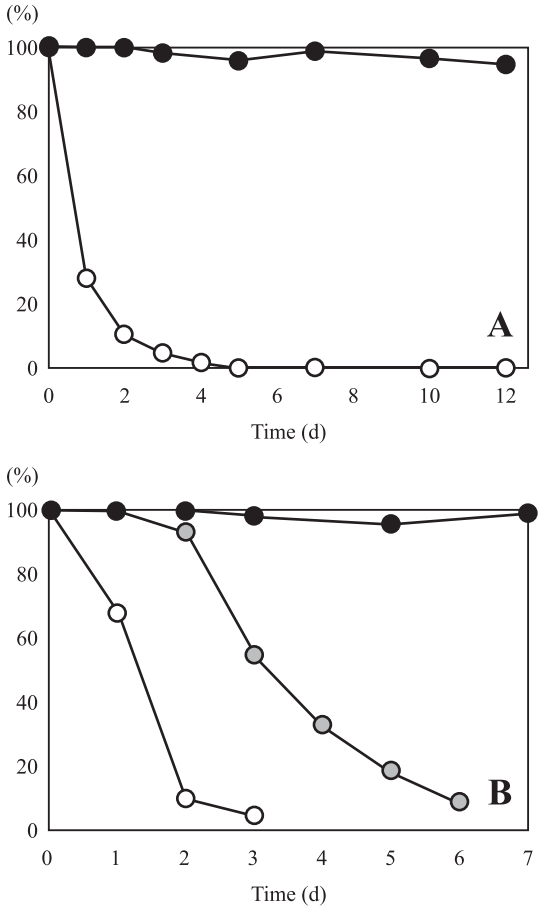

FIG. 3. Stability and curing of pRSS12 in *R. solanacearum* cells. (A) pRSS12-transformed MAFF106611 cells were cultured in CPG without kanamycin. Aliquots of the culture were taken at appropriate intervals for colony formation assay. The ratio of colony number on Km-containing plates to that on Km-minus plates is shown in percentage. For the control, assays with pKZ27-transformed MAFF106611 cells were carried out in parallel. Symbols: closed circles, pRSS12-transformed cells; open circles, pKZ27-transformed cells. (B) Curing of pRSS12 from transformed cells by EtBr treatment. pRSS12-transformed MAFF106611 cells were incubated in CPG with different EtBr concentrations (0, 3, and 10 μg/ml). At appropriate intervals, samples were taken for colony formation assay as described in panel A. Symbols: closed circles, 0 μg/ml EtBr; shaded circles, 3 μg/ml EtBr; open circles, 10 μg/ml EtBr.

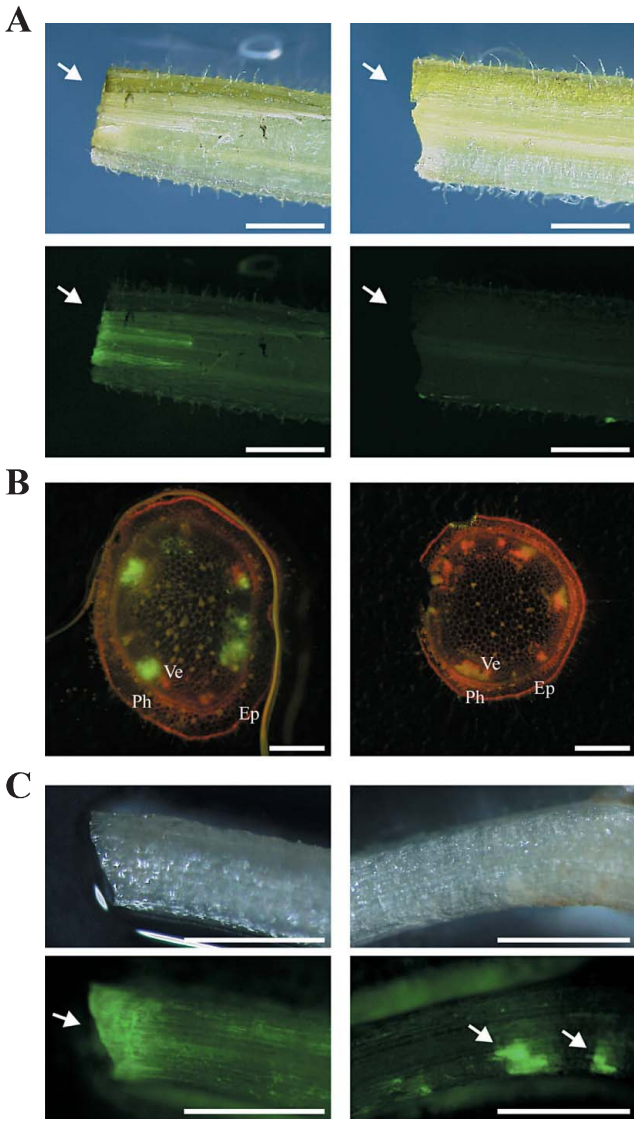

FIG. 4. *In planta* monitoring of pRSS12-transformed MAFF106611 cells. (A) Cells accumulated to a cut on a tomato petiole and penetrated into the leaf tissues via xylem vessels (12 h postinoculation). The bright-field (upper) and fluorescence (lower) images are shown. For control, nontransformed cells of MAFF106611 were added (right panels). Arrow indicates the cut edge. Bar represents 1 cm. (B) Accumulation of bacterial cells in the stem xylem vessels of a wilted tomato plant (wilting grade 3, 1 week postinfection). A cross section of the stem was cut 5 cm above the bacteria inoculation site on the tomato plant. Autofluorescence observed in the noninfected plant tissues is shown on the right panel for comparison. Ep, Epidermis; Ve, vessels; Ph, phloem. Bar marker represents 0.1 cm. (C) Cells accumulated to a cut on a tomato root and penetrated into the root tissues via xylem vessels (12 h postinoculation) (left panels). The bright-field (upper) and fluorescence (lower) images are shown. GFP-tagged cells also formed aggregates on the root surface (arrows on the right panel), from which bacterial invasion was also visible. Bar represents 1 cm.

cumulation of the bacterial cells to the xylem vessels in wilted tomato plants whose stem was injected with pRSS12-transformed cells (5×10^6 cells) was also markedly demonstrated in Fig. 4B. Almost the same responses (even with more extended degrees) were observed with a tomato root cut as shown in Fig. 4C. The cells grown at the edge at 12 h

postinfection invaded the root tissues along the xylem vessels (Fig. 4C, left panel). In addition to the cut edge, some dense aggregates were formed on the root surface (Fig. 4C, right panel), which were probably wounds or natural openings. The accumulation of bacterial cells in the xylem vessels of tomato roots was the same as that shown in Fig. 4B. These results indicated that pRSS12-transformed *R. solanacearum* cells could be easily detected in plant tissues and are useful in detailed cytological studies to reveal the infection mechanism of this serious phytopathogen.

Although there have been several reports on the expression of GFP-fused proteins in *R. solanacearum* cells so far, all the vectors used were selective-pressure-dependent (21, 22). For the constitutive expression of GFP in *R. solanacearum* cells, transposons have also been used: random chromosomal insertion of the pAG408 mini-transposon (23) was adopted to label the wild-type strain GMI1000 (22). To monitor the movement of individual cells and their chemotactic behaviors, Tn5-GFP-tagged *R. solanacearum* strains were examined (24, 25). However, there are intrinsic problems in using transposition techniques: Transposon insertion may affect the genetic background of host cells. The GFP expression itself may be affected by the genetic environment around the insertion site (position effects). Moreover, under natural environmental conditions with various physical and biological stresses, some transposons may be easily moved and lost (unstable).

Because pRSS12 can be easily introduced by electroporation and stably maintained in *R. solanacearum* cells of the different races and biovars, it serves as an easy-to-use GFP-tagging tool for any given *R. solanacearum* strain in the wild-type background. By monitoring pRSS12-transformed cells, the following may be studied in detail: (i) differences in the virulence traits among strains, (ii) differences in the resistance level (responses) of plant hosts against a given bacterial strain, (iii) effects of environmental factors during the infection establishment, and (iv) evaluation of therapeutic effects in the development of new agricultural chemicals for bacterial wilt disease.

Monitoring of *R. solanacearum* cells in soil samples by GFP fluorescence In the detection of *R. solanacearum* cells in cropping fields, some pretreatment or fractionation of soil samples would be required to release bacterial cells and to avoid background noises. To obtain preliminary information about cell recovery from soil samples, we performed brief fractionations of soil samples supplied with known numbers of pRSS12-transformed bacterial cells. Although no bacterial cells precipitated by centrifugation at 1000 rpm in a control containing only the culture fluid, the recovery of the labeled cells in the supernatant from soil samples was significantly low; only 17% of the cells in soil sample A and 36% in soil sample B (for soil samples A and B, see Materials and Methods) were recovered (measured by fluorescence counting) after centrifugation even at low speed (600 rpm for 5 min). This result clearly indicated that a significant portion of the bacterial cells easily adhered to soil particles. Because the two different soil samples showed different cell recovery values, the cell adhesion depended on the soil nature and condition. Therefore, for practical application, rigorous pretreatments of given soil samples should be performed to efficiently release adherent bacterial cells before the detection. A pilot experiment using different soil samples mixed with pRSS12-transformed bacterial cells may provide a good opportunity for this purpose and may yield useful information before application of various techniques to detect contaminating *R. solanacearum* cells in the cropping fields.

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