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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.

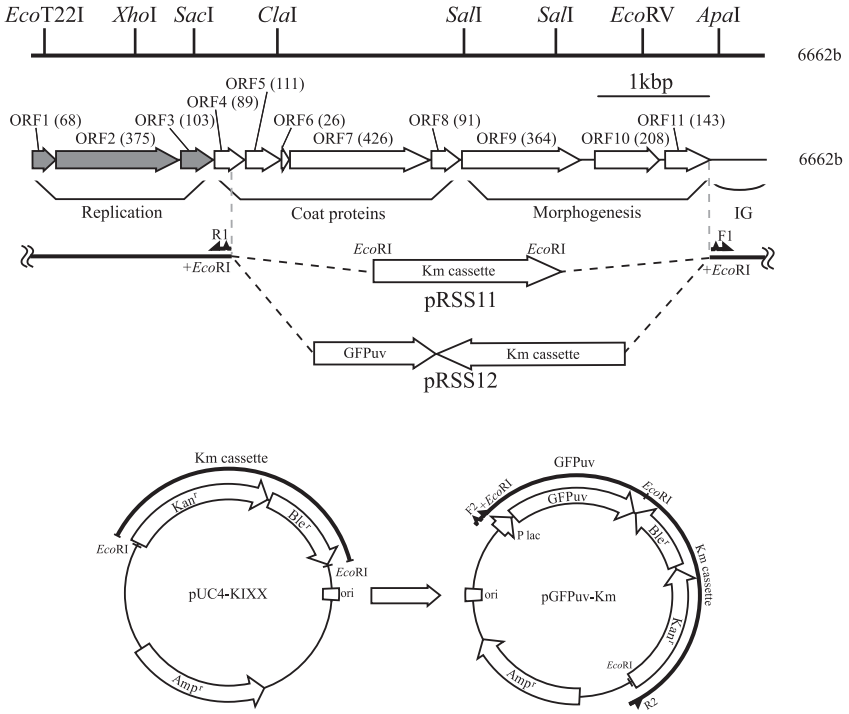

FIG. 1. Construction of pRSS12 from bacteriophage ϕRSS1 DNA. A portion containing ORF1-ORF3 and the intergenic region (IG) of ϕRSS1 DNA was amplified by PCR. This fragment was connected to a 1.5 kbp Km-cassette cut out from pUC4-KIXX with *Eco*RI, resulting in pRSS11 (6). The same ϕRSS1 fragment was 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.