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Monitoring growth and movement of *Ralstonia solanacearum* cells harboring plasmid pRSS12 derived from bacteriophage ϕRSS1

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We monitored growth and movement of *Ralstonia solanacearum* harboring the plasmid pRSS12 in tomato seedlings. The plasmid contains a gene for green fluorescent protein (GFP) and is stably maintained in *R. solanacearum* cells without selection pressure. Bacteria harboring the plasmid can be tracked *in planta* by visualizing GFP fluorescence. Stems of seedlings were infected with *R. solanacearum* cells transformed with pRSS12, and bacterial growth and movement, particularly around the vascular bundles, were monitored for more than 7 days. Our results showed that vascular bundles are independent of each other within the stem, and that it takes a long time for *R. solanacearum* cells to migrate from one vascular bundle to another. For real-time monitoring of bacteria *in planta*, tomato seedlings were grown on agar medium and bacterial suspension was applied to the root apex. The bacterial invasion process was monitored by fluorescent microscopy. Bacteria invaded taproots within 6 h, and movement of the bacteria was observed until 144 h after inoculation. In susceptible tomato cultivars, strong GFP fluorescence was observed in hypocotyls and lateral roots as well as the taproot. In resistant cultivars, however, GFP fluorescence was rarely observed on lateral roots. Our results show that this monitoring system can be used to assess bacterial pathogenicity efficiently.

[**Key words:** *Ralstonia solanacearum*; Bacterial wilt; Green fluorescent protein (GFP); Bacterial monitoring; Tomato (*Lycopersicon esculentum*)]

Ralstonia solanacearum is a soilborne gram-negative bacterium that causes bacterial wilt in many crops (1, 2). *R. solanacearum* cells infect roots, and exhibit tissue-specific tropism within the host, specifically invading and multiplying in the xylem vessels. In the field or in greenhouses, plants infected by the pathogen must be destroyed, and soil and draining systems must be sterilized with chemical bactericides. Because control of this pathogen has only limited success, bacterial wilt caused by *R. solanacearum* remains a serious economic problem in tropical and warm areas around the world (3). Therefore, researchers are striving to develop novel methods to control this pathogen. Recently, Yamada et al. (4) isolated and characterized various types of bacteriophage that specifically infect *R. solanacearum*. Such bacteriophages have potential uses as tools to control harmful pathogens, and for rapid and specific detection of *R. solanacearum* cells.

It is essential to have a rapid method to detect *R. solanacearum* cells so that the extent of bacterial infection and the effects of bactericides can be evaluated accurately. Conventional methods based on culturing bacteria isolated from plant tissue or soils on selective media are time consuming. It would be preferable to have a simple, real-time method for monitoring the pathogen *in planta*. Paraffin-embedded tissue sectioning and electron microscopy have been used for monitoring bacteria in plant tissues. Such methods are time- and labor-intensive, and involve complicated sample preparation procedures. Therefore, a simple and rapid real-time observation system is required. Hikichi et al. (5) reported monitoring of *R. solanacearum* cells transformed with the *lux* operon by detecting bioluminescence with a highly sensitive video camera. However, such equipment is expensive, and resolution of bioluminescence images is too low to observe bacterial growth and movement in plant tissues. There have been some reports on expression of green fluorescent protein (GFP) in *R. solanacearum* cells using plasmid vectors that are dependent on constant selection pressure (6). Such vectors are not well-suited for long-term monitoring in the plant body or in the soil without antibiotic selection, since plasmids are easily lost when the selection pressure is removed. For constitutive expression of GFP in *R. solanacearum* cells, random chromosomal insertions using mini-transposons were used (7) to label the wild-type strain GMI1000 (6). Tn5-GFP-tagged *R. solanacearum* cells were also examined (8). However, transposon insertion can affect the genetic background of host cells, and the expression of the inserted gene itself sometimes alters the genetic environment around the inserted genes. In an attempt to solve these problems, Kawasaki et al. modified the filamentous phage ϕRSS1 that specifically infects *R. solanacearum* (4) and developed a pRSS11 plasmid that is stably maintained in *R. solanacearum* cells without antibiotic selection pressure (9). pRSS11 was further modified and pRSS12 was developed to express GFP (10). pRSS12 was stably maintained over 100 generations without antibiotic selection (10), and was used in preliminary infection

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experiments to monitor bacterial growth and movement *in planta*. If transposon tagging is adopted for GFP-labeling, one can choose objective cell lines. This is an advantage. But, selection for the objective cell lines is time consuming. Transformation of *R. solanacearum* with plasmid pRSS12 is a very simple procedure. pRSS12 is a single-copy plasmid (10) and this eliminates effects induced by a multi-copy plasmid.

In this study, we investigated the growth and movement of *R. solanacearum* cells in plants' stems, particularly around junctions of vascular bundles, for more than 7 days. We then established a real-time monitoring system for bacterial growth using tomato seedlings grown on agar medium. This system can be used to rapidly evaluate susceptibility of tomato cultivars to bacterial infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions *R. solanacearum* strain MAFF 106611 was obtained from the National Institute of Agrobiological Sciences, Japan. The pRSS12-transformed MAFF 106611 cell line was established previously (10). Bacterial cells were cultured in CPG medium containing 0.1% casamino acids, 1% peptone, and 0.5% glucose (3) at 28 °C with shaking at 200–300 rpm.

Plants and culture conditions Seeds of tomato (*Lycopersicon esculentum*) cultivars, 'Oogata-fukujyu', 'Ponderosa', and 'B-barrier' were purchased from Takii Co., Ltd (Kyoto, Japan). For stem inoculations, tomato seeds were individually sown in pots containing a mixture of peat moss and expanded vermiculite at 25 ± 3 °C. Approximately 30 days after sowing, seedlings with four to six leaves were used for inoculation. For aseptic cultures, seeds were surface-sterilized with sodium hypochlorite and cultured in a square dish (sterile square schale No. 2; Eiken Chemical Co., Ltd, Tokyo, Japan) containing solid medium (0.15% Hyponex powder (Hyponex Japan Corp., Ltd., Osaka, Japan), 0.5% sucrose, and 1.5% agar adjusted to pH 5.8). Plants were grown in a growth chamber at 28 °C under a 16 h light/8 h dark photoperiod for 7 to 21 days. During the culture period, the dishes in the chamber were tilted to a 45° angle to encourage roots to grow along the surface of the medium.

Inoculation to plants To inoculate into plants, bacterial cells were cultured in CPG medium, and suspended in sterile distilled water at a density of 1×10^8 cells/ml. The bacterial suspension was injected with a needle into the second internode on the major stem of 4-week-old tomato plants (with 4–6 leaves). After inoculation, plants were cultured in the growth chamber as described above. To observe bacteria in the stems, thin sections of the stems were prepared by hand, and were observed using an MZ16F fluorescence stereomicroscope (Leica Microsystems, Heidelberg, Germany) equipped with GFP2 and GFP3 filters and/or an Olympus BH2 fluorescence microscope (Olympus, Tokyo, Japan). To inoculate into plants grown in culture dishes, the tip of the taproot was cut with a razor blade at 10 mm from the apex, and then 2 µl of bacterial suspension was applied to the section. After inoculation, the plants in the dishes were cultured in the growth chamber until observation. After inoculation, growth and movement of the bacterial cells were observed under the fluorescence stereomicroscope for 168 h. Microscopic images were recorded with a CCD camera (Keyence VB-6010; Osaka, Japan).

Measurement of GFP fluorescence intensity Cells of *R. solanacearum* strain MAFF 106611 harboring pRSS12 and without pRSS12 were cultured in CPG medium, and log-phase cells were collected. The cells were washed with distilled water, and re-suspended in distilled water at the density of 1.5×10^8 cells/ml. Then, serial dilution series of each cell line were prepared. Fluorescence intensities of GFP were measured with Infinite M200 micro plate reader (Tecan, Männedorf, Switzerland) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Monitoring of *R. solanacearum* in plant stems Previously, we reported that bacterial dynamics within stems of tomato plants could be monitored using pRSS12 (10). In this study, we used pRSS12 to visualize the connectivity of xylem vessels within the stem. After inoculation, *R. solanacearum* cells moved in the plant body mainly through xylem vessels. However, the bacterial cells did not always spread throughout the entire plant (our unpublished data). Xylem

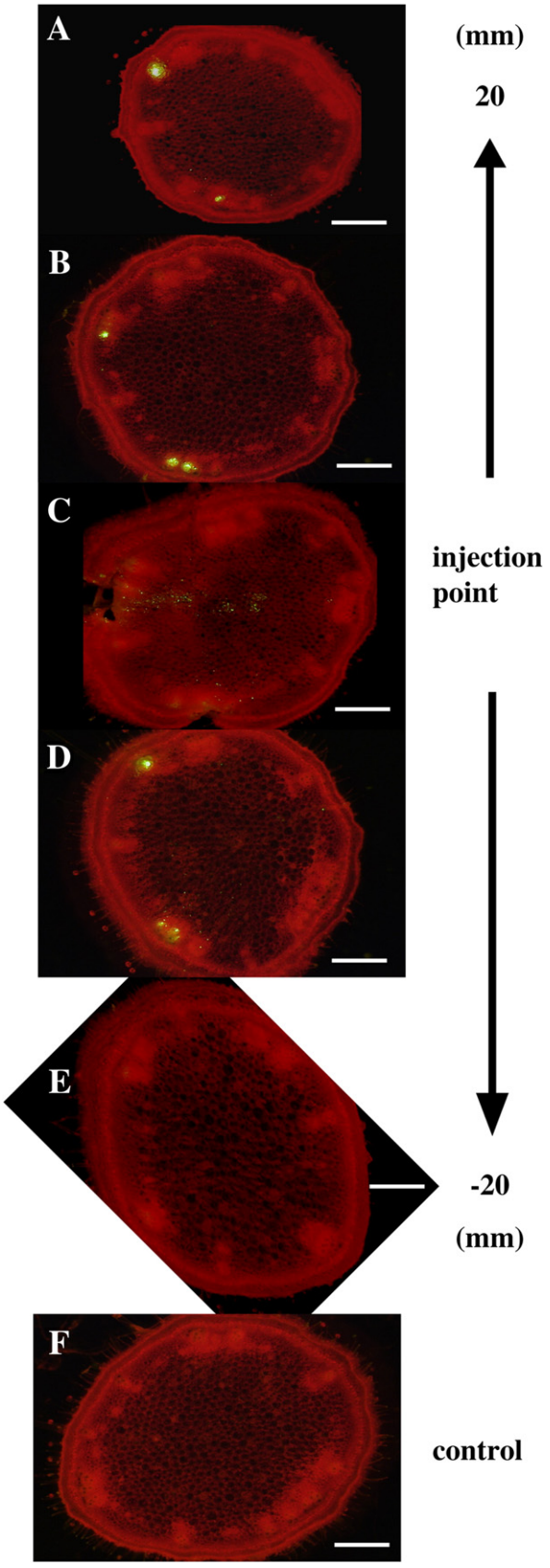

FIG. 1. Cross sections of tomato seedlings inoculated with *R. solanacearum*. Green spots are GFP signals. Red fluorescence is auto-fluorescence from chlorophyll. (A) 20 mm above injection point; (B) 10 mm above injection point; (C) injection point; (D) 10 mm below injection point; (E) 20 mm below injection point. Bars = 1 mm.