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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

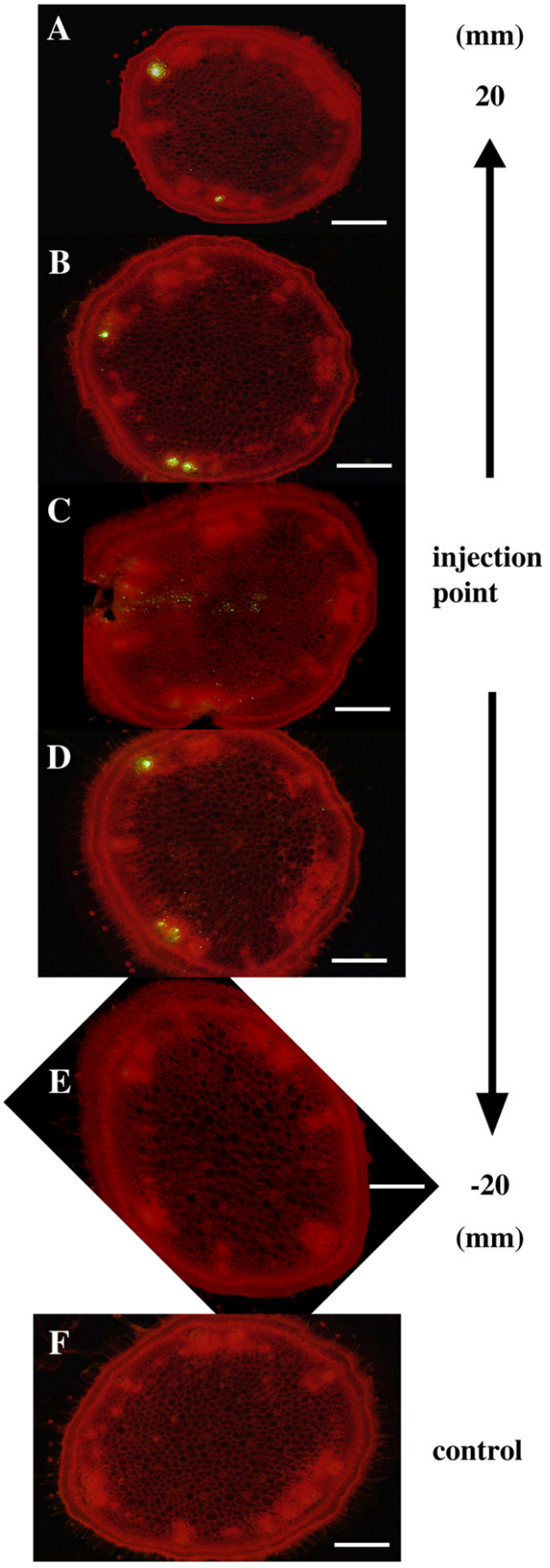

FIG. 1. Cross sections of tomato seedlings inoculated with *R. solanacearum*. Green spots are GFP signals. Red fluorescence is auto-fluorescence from chlorophyll. (A) 20 mm above injection point; (B) 10 mm above injection point; (C) injection point; (D) 10 mm below injection point; (E) 20 mm below injection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

system in the plant body is an aggregate of variable length of vessels (11). At the points where xylem vessels elements are connected, water is mobilized via the pit, apoplastic pathway and/or symplastic pathway, but the movement of bacteria may be delayed because of their large size. In this study, we selected *R. solanacearum* strain MAFF 106611 to study bacterial movement in tomato stems because of its consistent infectivity. We selected the tomato cultivar 'Oogata-fukujyu' because it is sensitive to infection by *R. solanacearum*, and because it has a low level of auto-fluorescence, which results in increased resolution of microscopic images. Stems of tomato were injected with pRSS12-transformed *R. solanacearum* cells, and movement of the transformed cells was investigated by analyzing cross sections (Fig. 1) taken at 10-mm intervals from the inoculation point 5 days post inoculation (p. i.). After 3–5 days p. i., cross sections were taken at 10-mm intervals from the inoculation point. The length of bacterial movement during the period was determined as the distance between the inoculation point and the farthest section where GFP signals were observed. The cells moved upward from the inoculation point at a speed of 0.4 to 0.8 mm/h, and moved downward at a speed of 0.1 to 0.2 mm/h. The difference between the upward and downward movement speeds reflects the upward movement of water through the xylem vessels. We confirmed that pRSS12-tranformed cells retain their fluorescence even 6 days after inoculation by observing cross sections with an epi-fluorescence microscope (our unpublished data).

We then examined the xylem system junctions at the base of petioles. At 4 to 6 days p. i., we investigated accumulation of *R. solanacearum* cells close to the xylem junctions. Cells of *R. solanacearum* were inoculated to the stem below the petiole junctions (Fig. 2A). The cells were injected just below the petiole (for Fig. 2B, C) or opposite side of the petiole (for Fig. 2D, E). The pRSS12-transformed cells had successfully traveled to just below the petiole junction (Fig. 2B, D). When labeled bacteria entered the petiole, there were no fluorescent signals observed in vascular bundles in the stem above the petiole junction (Fig. 2C). When the bacteria did not enter the petiole, labeled cells were visible in the vascular bundle. These results indicate that vascular bundles in the tomato stem are independent of each other, and that *R. solanacearum* cells cannot migrate to different vascular bundles in a short period of time. The fact that the xylem vessels are discontinuous may be one reason why bacterial cells are not found throughout the entire plant body, even in severely wilted plants.

Infection of *R. solanacearum* to seedlings grown on solid agar medium We monitored real-time bacterial dynamics in inoculated plants grown on solid agar medium. Because microscopic resolution declines markedly when roots penetrate into the medium, we grew plants in conditions that encouraged the growth of roots along the medium's surface (1.5% agar, with plates tilted at a 45° angle). Plants were inoculated by cutting the root tip with a razor blade and applying 2 μl of bacterial suspension (1×10^8 cells/ml; Fig. 3A). In these conditions, more than 95% of seedlings were infected with bacterial cells and GFP fluorescence was observed in the taproots (data not shown). When 7-day-old seedlings of the tomato cultivar 'Oogata-fukujyu' were inoculated, GFP signals in the roots were clearly observed at 6 h p. i. (Fig. 3B), and the bacterial cells moved more than 30 mm from the inoculation points at 12 to 24 h p. i. (Fig. 3C) at a speed of 1 to 4 mm/h. GFP signals were observed in the hypocotyls 24 to 48 h p. i. (Fig. 3D). Bacterial cells invaded lateral roots within 6 to 48 h after reaching the root junctions (Fig. 3E). No GFP signals were observed when plants were inoculated with *R. solanacearum* cells that did not harbor pRSS12 (Fig. 3F). Our results indicate that this method is a rapid and simple way of observing behavior of GFP-labeled *R. solanacearum* cells *in planta*.

The root of seven-day-old 'Oogata-fukujyu' seedling was inoculated with *R. solanacearum* strain MAFF 106611 harboring pRSS12 and change of GFP fluorescence was monitored until 24 h p. i. (Fig. 3G, H, I).

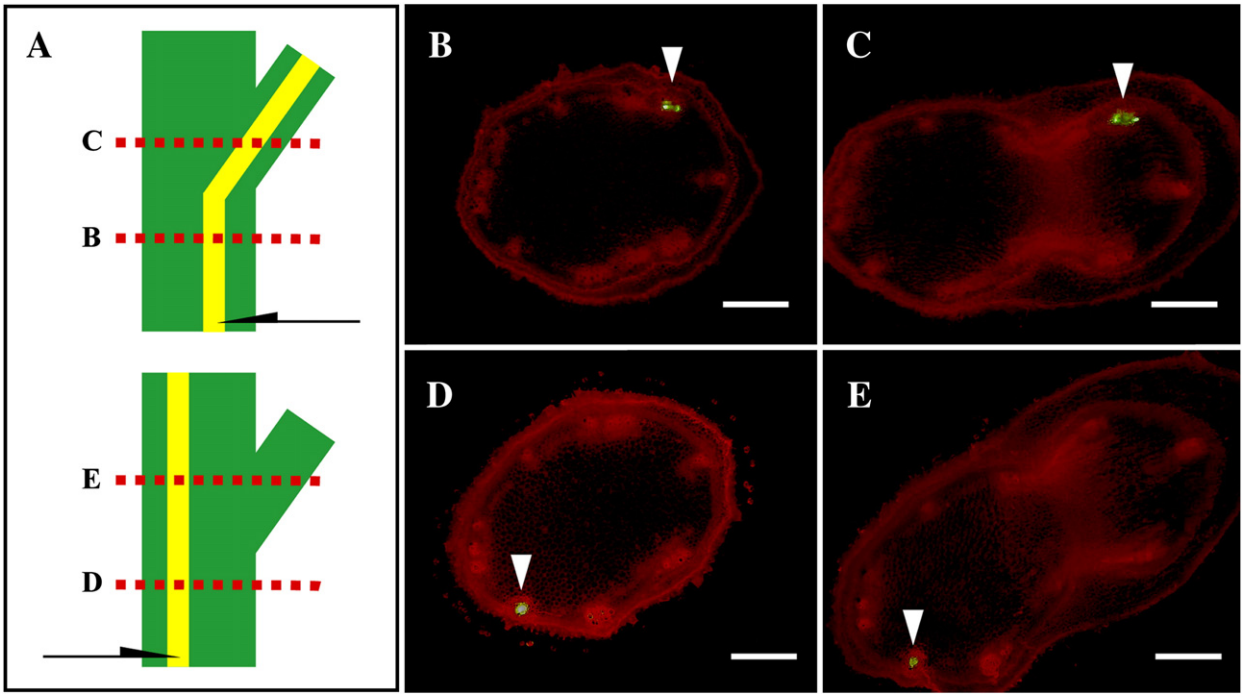

FIG. 2. Cross sections of tomato seedlings 4–6 days after *R. solanacearum* inoculation. Green spots are GFP signals. Red fluorescence is auto-fluorescence from chlorophyll. (A) Schematic view of *R. solanacearum* inoculation in the stem. Yellow lines indicate vascular bundles. Cross sections were prepared at B–E. Arrows show injection points of GFP-labeled *R. solanacearum* cells. (B) Stem below the petiole junction. (C) Section at 5 mm above the petiole junction. (D) Stem below the petiole junction. (E) Section at 5 mm above the petiole junction. (B, C) Infected vascular bundles are lead to the petiole; (D, E) infected vascular bundles are not lead to the petiole. Arrowheads in B–E show GFP signals. Bars = 1 mm.

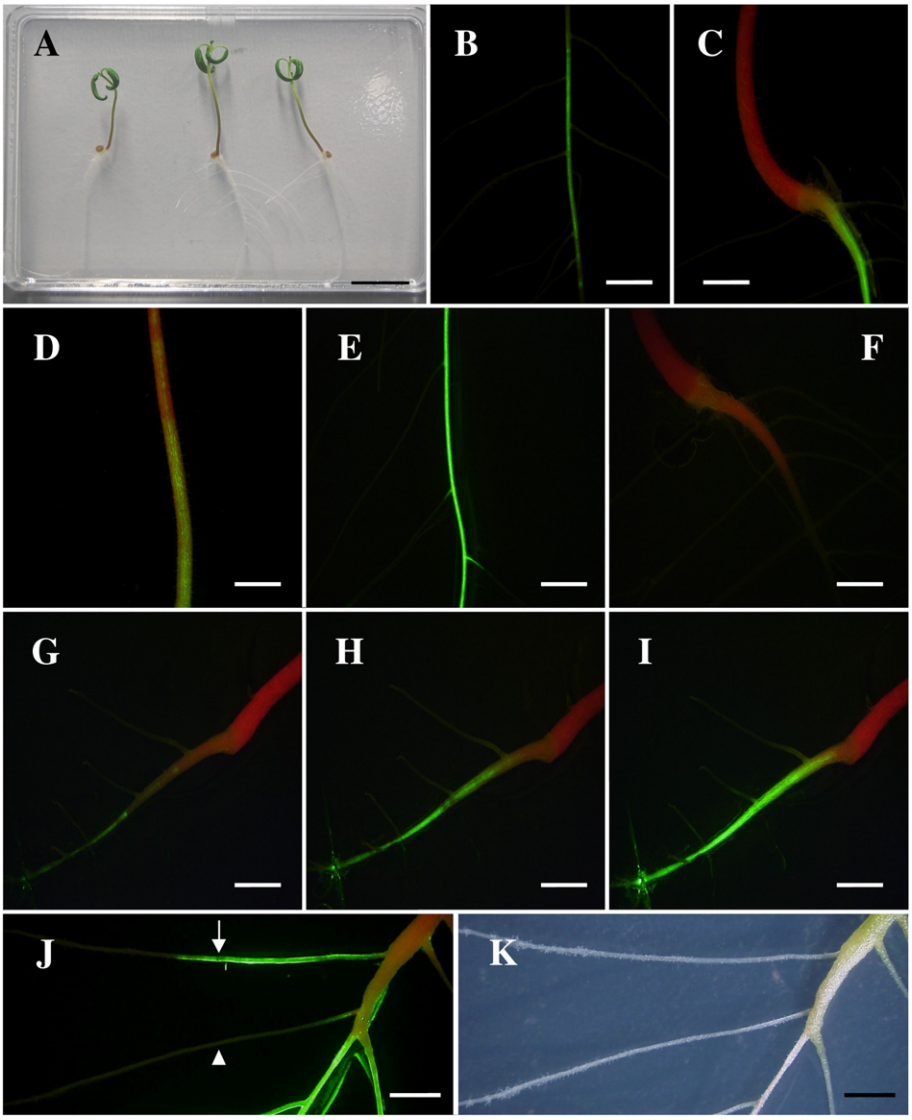

FIG. 3. Infection of seedlings grown on solid agar medium. (A) Seven-day-old seedlings of 'Oogata-fukujyu' before inoculation. Bar in panel (A) = 2 cm. (B) Fluorescence in inoculated roots after 12 h; (C) boundary of root and hypocotyl after 24 h; (D) infected hypocotyls; (E) lateral roots 48 h after inoculation; (F) seedlings inoculated with *R. solanacearum* cells not harboring pRSS12. (G–I) Time course analysis of bacterial growth inoculated to the root of 'Oogata-fukujyu' seedling. Seven-day-old seedling was inoculated with *R. solanacearum* strain MAFF 106611 harboring pRSS12 and change of GFP fluorescence was monitored. (G) 12 h p. i. (H) 18 h p. i. (I) 24 h p. i. (J, K) Lateral roots of 'B-barrier' seedling 120 h p. i. Lateral root with strong fluorescence (arrow) and lateral root with weak fluorescence (arrowheads) are used to study the bacterial population in the roots. (K) Bright field image. Bars in B–K = 2 mm.

After inoculation, fluorescence intensity increased in 24 h. Increase of GFP fluorescence after inoculation seems to support bacterial growth and movement in the root after inoculation.

Relation between GFP fluorescence intensity and bacterial population Relation between fluorescence intensity and bacterial density was examined (Table 1). Fluorescence intensity of *R. solanacearum* strain MAFF 106611 harboring pRSS12 was dependent on the cell density significantly. Weak fluorescence of the cells not harboring the plasmid may be auto-fluorescence. The relation between fluorescence intensity and bacterial population in the infected roots was also examined (Fig. 3J, K). The lateral roots with strong fluorescence and weak fluorescence shown in Fig. 3J were cut from the taproots and 15 mm-long sections were prepared. Each section was homogenized in sterile water and the number of released bacteria was counted by plating diluted homogenate on CPG plate media containing kanamycin (50 μg/ml). The numbers of released cells were 3.1×10^6 cells/mm^3 and 1.2×10^5 cells/mm^3 from the lateral roots with strong fluorescence and with weak fluorescence, respectively. This observation shows that fluorescence intensity of GFP in the plant organ also depends on the bacterial population.

Susceptibility of different tomato cultivars to *R. solanacearum*
The tomato cultivars 'Oogata-fukujyu' and 'Ponderosa' are susceptible to *R. solanacearum* strain MAFF 106611 infection, and the cultivar 'B-barrier' is resistant. When 7-day-old seedlings were inoculated, GFP fluorescence was detected clearly on the taproots, on the lateral roots and on the hypocotyls in all of the three cultivars. However, in the

TABLE 1. Relations between fluorescence intensity and bacterial density.

Cell density (cells/ml)	1.5×10^8	1.5×10^7	1.5×10^6	1.5×10^5	1.5×10^4	1.5×10^3
(+) pRSS12	45922	4225	510	156	135	127
(−) pRSS12	373	154	117	113	110	110
DW	103	117	117	121	117	117

Values are unit of fluorescence intensity. (+) pRSS12: *R. solanacearum* strain MAFF 106611 harboring pRSS12. (−) pRSS12: *R. solanacearum* strain MAFF 106611 not harboring pRSS12. DW: distilled water (control).

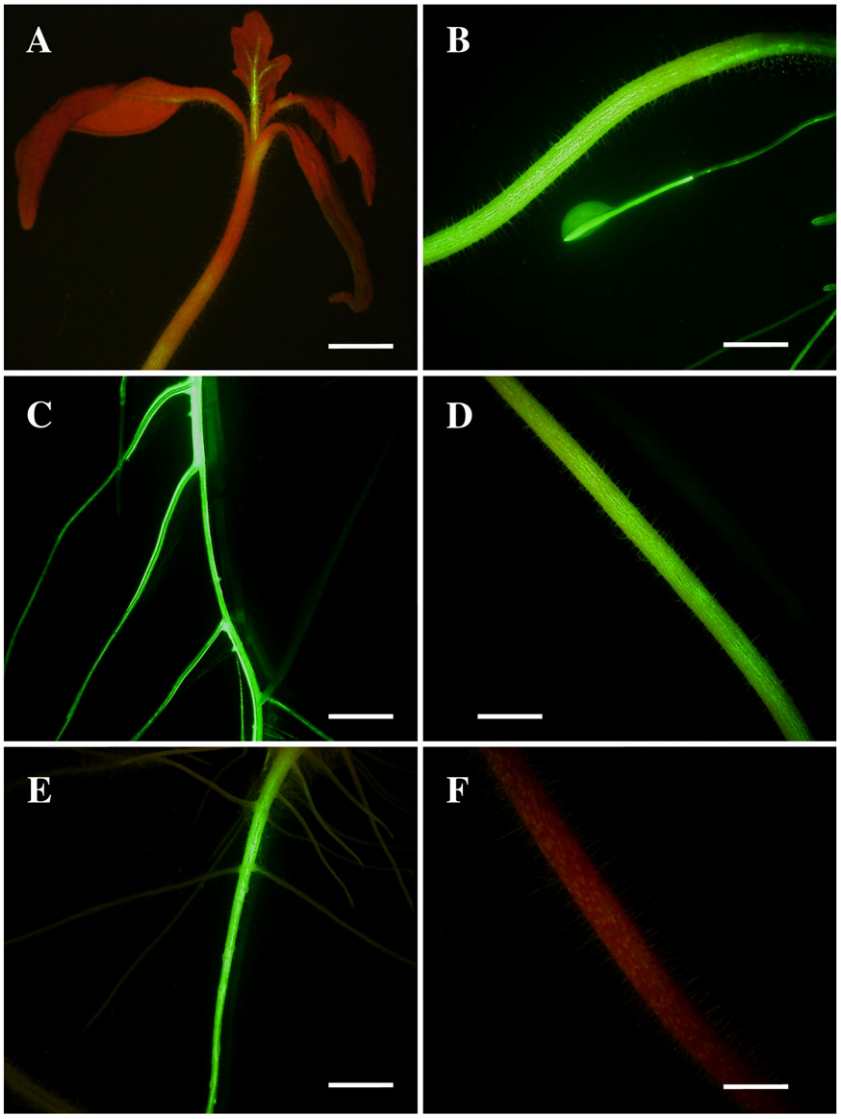

FIG. 4. Infection process in susceptible and resistant cultivars. (A) Seven-day-old seedling of 'Ponderosa' (susceptible cultivar). GFP fluorescence is visible in leaf vein. (B) Bacteria leaking from root apex of 7-day-old 'Ponderosa' seedling. Root (C) and hypocotyl (D) of 'Oogata-fukujyu' (susceptible cultivar) at 72 h p. i. Strong GFP signals were detected. Taproot (E) and hypocotyl (F) of 'B-barrier' (resistant cultivar) at 120 h p. i. GFP signals were scarcely observed on the lateral roots, and on the hypocotyl. Bars = 2 mm.

susceptible cultivars, *R. solanacearum* cells sometimes entered leaf veins (Fig. 4A) and leaked from the apex of lateral roots (Fig. 4B). When 21-day-old seedlings were inoculated, there were clear differences between susceptible and resistant cultivars. When 'Oogata-fukujyu' was inoculated, strong GFP fluorescence was observed in the taproots, lateral roots, and hypocotyls at 72 h p. i. (Fig. 4C and D). A similar pattern of fluorescence was observed in 'Ponderosa' (data not shown). However, when 'B-barrier' was inoculated, there was rather weak fluorescence in the taproot, and lateral roots were rarely infected (Fig. 4E; Table 2). GFP fluorescence signals from hypocotyls were very weak (Fig. 4F), even at 96 h p. i.

TABLE 2. Infection of lateral roots in 21-day-old seedlings.

Time after inoculation	72 h	90 h	108 h
'Ponderosa'	9/12	11/12	12/12
'Oogata-fukujyu'	9/16	11/16	16/16
'B-barrier'	2/14	2/14	3/14

Figures show the ratios of lateral roots where GFP signals were observed / total numbers of lateral roots of each seedlings observed.

One of the differences between resistant and susceptible cultivars found in this study was the invasion of lateral roots. This difference may be due to gaps between the taproot xylem and the lateral root xylem. It appears that *R. solanacearum* cells require a long period of time to move between xylem gaps in resistant cultivars. We also observed that while there was some increase of GFP signal intensity in roots of the resistant cultivar 'B-barrier', GFP signal intensity was not obvious in the hypocotyl and stem. This means bacterial growth was suppressed in the hypocotyl and stem of the seedlings of cultivar 'B-barrier'. These observations are consistent with the studies of Nakaho (12) and Hikichi et al. (5) using grafted plants. In our study, fluorescence intensity was stable even until 144 h after infection. This is a characteristic of pRSS12, which is stably maintained in *R. solanacearum* cells without selective pressure.

Our results indicate that this system, using bacterial cells expressing pRSS12, is suitable for monitoring bacterial growth and movement *in planta*. We monitored bacterial infection behavior in plants grown on solid agar medium. This system is simple and reproducible, and enabled rapid observation of bacterial infection (as

soon as 12 h p. i.). This is more rapid than other methods of monitoring disease development, e.g., calculating disease index from wilting of pot-grown plants several days after inoculation (3). Aseptic inoculation of plants grown on solid agar medium eliminates the effects of other bacteria in the soil. The culture dishes are compact, which means that more samples can be used than in traditional pot-based methods. Our results show that this method is an effective way of monitoring growth and movement of *R. solanacearum* within the plant body and it could be an efficient tool for the development of bactericides.

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