Spatial-Temporal and Quantitative Analysis of Growth and EPS I Production by *Ralstonia solanacearum* in Resistant and Susceptible Tomato Cultivars

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ABSTRACT

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One susceptible and two resistant cultivars of tomato were tested for differences in infection by *Ralstonia solanacearum* and for the subsequent multiplication, colonization, and production of the wilt-inducing virulence factor, exopolysaccharide I (EPS I). Bacterial ingress into the taproot was fastest in the susceptible cv. Marion, followed by the resistant cvs. L285 (fivefold slower) and Hawaii 7996 (15-fold slower). Once inside the taproot, *R. solanacearum* colonized, to some extent, almost all regions of the resistant and susceptible plants. However, colonization occurred sooner in the susceptible than in the resistant cultivars, as measured by viable

cell counts of bacteria in the midstems. Rates of multiplication and maximum bacterial cell densities were also greater in the susceptible than in the resistant cultivars. Growth experiments utilizing xylem fluid from infected and uninfected plants indicated that neither antimicrobial activities nor reduced levels of growth-supporting nutrients in the xylem fluids were responsible for the reduced bacterial multiplication in the resistant cultivars. Quantification of EPS I in the infected plants, using an enzyme-linked immunosorbent assay, revealed that the bacterial populations in the susceptible cultivar produced greater amounts of EPS I per plant than those in the resistant cultivars. Immunofluorescence microscopy using antibodies against either EPS I or *R. solanacearum* cells revealed that bacteria and EPS I were distributed throughout the vascular bundles and intercellular spaces of the pith in the susceptible cultivar, whereas in the resistant cultivars, bacteria and EPS I were restricted to the vascular tissues.

Ralstonia (Pseudomonas) solanacearum (42,43) is a soilborne phytopathogenic bacterium that causes a lethal wilting disease in over 200 different species of plants including the economically important crops tomato, potato, eggplant, and banana (26). Bacterial wilt disease occurs throughout tropical and subtropical regions and has reduced the yields of important crops (22). Root infection by *R. solanacearum* occurs through wounds caused by various agents such as nematodes, insects, agricultural equipment, and the destructive outgrowth of secondary roots (27,36). Vasse et al. (39) described a three-stage process in which *R. solanacearum* could infect undisturbed tomato roots grown in a hydroponic system. In their model, the pathogen initially colonized the exterior of the root, especially at the elongation zone and the sites of secondary root emergence. The bacteria then colonized the intercellular spaces of the cortex, infected the vascular parenchyma, and finally invaded the xylem vessel elements. *R. solanacearum* entered the vessels either by directly penetrating the vessel element walls or by release from tyloses formed by the contact cells (39,40). Once in the xylem vessels, the bacteria rapidly spread throughout the plant, resulting in wilt symptoms and death of the host plant.

Several virulence factors produced by *R. solanacearum* have been identified, including plant cell wall-degrading enzymes and exopolysaccharide I (EPS I). Plant cell wall-degrading enzymes appear to enhance virulence by promoting invasion and vascular colonization (1,25,35); however, none of these enzymes are absolutely required for disease. In contrast, EPS I (a high-molecular-weight, nitrogen-rich, acidic exopolysaccharide [32]) is essential for wilting and killing of host plants (9,30). EPS I is produced in copious amounts both in planta and in vitro and is believed to function by

clogging the xylem vessels, resulting in wilt. EPS I has also been shown to aid in the systemic colonization of susceptible tomato cultivars (3,35).

R. solanacearum persists in the soil (11,14,24), the rhizosphere of weeds (10), or in the roots of nonhost plants (14,37) for extended periods of time. Attempts to eliminate it from infested soils using rotation or fumigation have been either ineffective or too expensive (12). Thus, the most promising control strategy has been the development of resistant cultivars (6,22). Tomato cvs. Hawaii 7996 and L285 have been shown to be highly resistant to bacterial wilt disease in both greenhouse and field studies (16,19–21). Resistance in both cultivars is a complex trait involving multiple genes located on different chromosomes (8,38). In cv. Hawaii 7996, putative quantitative trait loci have been mapped to chromosomes 3, 4, 6, 8, 10, and 11 (38); likewise, in cv. L285, resistance loci have been mapped to chromosomes 6, 7, and 10 (8). Although the genes responsible for resistance have not been cloned and characterized, it is speculated that the resistance loci on chromosome 6 are the same in both cultivars due to their proximity to the nematode resistance gene *Mi* and their dominant contribution to resistance (8, 38). Phenotypic evaluation of wilt resistance in cv. Hawaii 7996 has been previously conducted by inoculating *R. solanacearum* onto wounded roots and monitoring wilt symptom development and colonization (15–17). These studies indicated that resistance does not arise from an inability of the bacteria to invade the roots, but rather from a limitation of their spread from the collar to the midstem. Little information is available about the resistance in cv. L285, as most studies have concentrated on mapping resistance loci (8,20).

Although a few studies have investigated the mechanisms by which resistant tomato cultivars avoid wilt caused by *R. solanacearum*, none have carefully analyzed colonization in conjunction with in planta immunolocalization and quantification of the major virulence factor, EPS I. Here, we report novel data about the penetration of unwounded roots, the colonization of aerial tissues, and the production of EPS I by *R. solanacearum* in both resistant and

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susceptible tomato cultivars. We also present immunofluorescence microscopy data that demonstrates differences in the location of bacteria and EPS I in resistant and susceptible cultivars.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *R. solanacearum* AW1-10A is a nalidixic acid and kanamycin-resistant wild-type strain that constitutively produces β -galactosidase (35). The *R. solanacearum* *hrp* mutant used was GM1475 (4). *R. solanacearum* was routinely grown on BG agar (1% peptone, 0.1% yeast extract, 0.1% casamino acids, 0.5% glucose, and 1.8% agar) (7); the minimal medium used was basal salts medium (BSM) (34). All media were supplemented with nalidixic acid (20 μ g/ml) and kanamycin (50 μ g/ml). BGC (BG plus crystal violet [25 μ g/ml], cycloheximide [50 μ g/ml], nalidixic acid [20 μ g/ml], and kanamycin [50 μ g/ml]) was used for monitoring bacterial growth in plant tissues. To prepare inocula, bacteria were grown on BG agar at 30°C for 2 days and then a single mucoid colony was transferred to BG broth and shaken overnight at 30°C. This culture was diluted 100-fold into BSM and shaken until the optical density at 600 nm (OD_{600}) = 1.0; bacteria were then washed in distilled water and resuspended to the appropriate OD_{600} .

Plant cultivars, growth conditions, and inoculation. Tomato cultivars used were the susceptible cv. Marion (35) and the resistant cvs. L285 (21) and Hawaii 7996 (15,41). Plants were grown in Styrofoam cups as described previously (35). Petiole inoculations were performed by excising the first petiole above the cotyledons 5 mm from the stem and depositing a 10- μ l drop of a bacterial suspension containing 10^8 or 10^3 bacteria on the petiole stump. The plants were held in a growth chamber and harvested on day 2 (no wilt symptoms in the susceptible cultivar), day 5 (onset of wilt in the susceptible cultivar), or day 8 (total wilt of the susceptible cultivar). Root inoculations were performed by pouring 20 ml of a bacterial suspension onto the soil to give a final concentration of 10^8 bacteria per g wet weight. In bacterial ingress experiments, 20 ml of a bacterial suspension containing 5×10^8 bacteria per ml was poured onto the soil. The plants were returned to the growth chamber and harvested as described below.

Xylem fluid was obtained by cutting the stem of 5-week-old tomato plants at the hypocotyl and allowing the xylem fluid to accumulate on the cut surface for 20 min. The xylem fluid was removed with a pipette, sterilized using a 0.2- μ m syringe filter, and inoculated with approximately 10^3 bacteria per ml. After shaking at 30°C for 24 h, bacteria were quantified by viable plate counts. Xylem fluid of infected plants was obtained similarly, except that plants were petiole-inoculated with 10^3 bacteria 24 h before harvesting xylem fluid.

Sampling and analysis. For soil infestation experiments, plants were harvested and quantified for bacteria and EPS I by removing the Styrofoam cups and washing the roots with water until the soil was removed. The roots were immersed in 1% sodium hypochlorite for 1 min and rinsed with tap water. The roots and stems were sprayed with 80% ethanol, which was then burned off. The lateral roots were removed with a razor blade, the primary root was excised, and a 3-cm cross section was taken. Stem samples (3 cm) were taken at the cotyledon and 15 cm above the cotyl node (midstem). The first two petioles were sampled by excising them from the stem and removing the leaves.

Samples were weighed, placed in 10 ml of phosphate-buffered saline (PBS) (50 mM potassium phosphate buffer, pH 7.0, and 150 mM NaCl) per g and homogenized with a PowerGen 700 tissue homogenizer (Fisher Scientific Co., Pittsburgh) at 4°C for 10 s on power setting 5. Petiole-inoculated plants were processed in the same manner, except that 3-cm samples of the stem were taken at the site of inoculation and also 10 cm above and below the site of inoculation. Bacteria were enumerated by serial dilution of the homogenates in PBS and plating onto BGC agar. EPS I was quanti-

Fig. 1. Bacterial multiplication in tomato plants grown in infested soils. Three plants of the susceptible cv. Marion (filled circles), and the resistant cvs. Hawaii 7996 (open squares) and L285 (open circles) were soil-inoculated to a final concentration of 10^8 bacteria per g of soil. Sections (2 cm) from the **A**, taproot; **B**, hypocotyl; **C**, lowest two petioles; and **D**, midstem were removed, processed, and the bacteria in them enumerated by viable counts as described in Materials and Methods.

fied by an enzyme-linked immunosorbent assay (ELISA) (28,30) using the mouse immunoglobulin (Ig) M monoclonal antibody Ps1a (2; A. Alvarez, *personal communication*). This monoclonal antibody is highly specific for EPS I produced by *R. solanacearum*, as it does not react with polysaccharides produced by *Rhizobium meliloti*, *Xanthomonas campestris*, *Escherichia coli*, or *R. solanacearum* mutants blocked in the EPS I biosynthetic operon (*eps*) (30). The secondary antibody used in the ELISA was goat anti-mouse IgM conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA).

Immunofluorescence microscopy. Segments of root, petiole, and stem were cut into 5-mm sections and fixed for 1 h in 3% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2). Samples were washed three times in 0.2 M sodium cacodylate buffer, dehydrated in a graded series of ethanol-water (1-h intervals in 15, 30, 45, 75, 95, and 100% ethanol), infiltrated with Immuno-Bed infiltration solution (Polysciences, Inc., Warrington, PA), and polymerized at 4°C in the presence of 100% N₂ gas. Specimens were cut into 3-μm-thick sections using a microtome, transferred to a pool of water, mounted on glass slides, and allowed to dry. Sections were washed with 100% ethanol, rehydrated in a graded series of ethanol to 100% water (1-min intervals in 15, 30, 45, 75, 95, and 100% water), incubated for 30 min in 2% bovine serum albumin fraction V (BSA) (Sigma Chemical Co., St. Louis) in TBST buffer (20 mM Tris [pH 7.4], 0.85% NaCl, 0.1% BSA, and 0.05% Tween 20) at 25°C, washed three times in PBS, and incubated overnight at 4°C with primary antibodies (mouse anti-β-galactosidase IgG, Promega Corp., Madison, WI; or mouse anti-EPS I IgM [2]) in TBST. The slides were washed three times with TBST and incubated with goat anti-mouse IgG fluorescein isothiocyanate conjugate or goat anti-mouse IgM rhodamine conjugate (Jackson ImmunoResearch Laboratories) in TBST for 1 h at 25°C. Sections were washed with TBST, mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA) and examined with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

RESULTS

Bacterial multiplication and EPS I production in plants grown in infested soils. These experiments were performed to examine the ability of *R. solanacearum* to invade the roots and colonize the susceptible cv. Marion and the resistant cvs. L285 and Hawaii 7996. Bacteria invaded the roots and spread to the hypocotyl and lowest petioles of both the resistant and susceptible cultivars within 2 days (Fig. 1A to C). However, the apparent rate of bacterial increase in the susceptible cv. Marion (10⁷ bacteria per day) was 10-fold and 100-fold greater than that in cvs. L285 and Hawaii 7996, respectively. Likewise, the maximal bacterial cell densities were dramatically less in cvs. Hawaii 7996 and L285 than that in cv. Marion. In the hypocotyl of cv. Marion, the average maximal bacterial density was 2.7×10^8 bacteria per g of fresh plant tissue (b/gpt), whereas it was only 3.6×10^6 b/gpt in cv. L285 (>70-fold reduction) and 8.5×10^4 b/gpt in cv. Hawaii 7996 (>3,000-fold reduction). The petioles and midstems of the resistant cultivars contained even fewer bacteria, and the differences in populations between cv. Marion and the resistant cultivars in these regions were even greater (Fig. 1C and D).

Despite the fact that bacterial population densities reached 10⁷ b/gpt in the roots and hypocotyls of cv. L285, no disease symptoms developed in either resistant cultivar. This behavior resembled that of EPS I-deficient *R. solanacearum* colonizing the susceptible cv. Marion (35). Therefore, it was important to quantify the amount of EPS I produced in both the resistant and susceptible cultivars. EPS I production in the root, hypocotyl, and midstem during the first 5 days was similar in cvs. L285 and Marion, with bacteria in both cultivars producing approximately 10 μg of EPS I per g of fresh plant tissue (μg/gpt) (Fig. 2A, B, and D). After day 5, EPS I production

Fig. 2. EPS I production in tomato plants grown in infested soils. Three plants of the susceptible cv. Marion (filled circles), and the resistant cvs. Hawaii 7996 (open squares) and L285 (open circles) were soil-inoculated and processed as in Figure 1, and the EPS I in sections was quantified by enzyme-linked immunosorbent assay.

in these regions stopped in cv. L285, whereas in cv. Marion, EPS I levels increased another 10-fold. In contrast, EPS I production by *R. solanacearum* colonizing cv. Hawaii 7996 was very low and never exceeded 1 µg/gpt at any site sampled. After the bacterial populations reached their maximum in the taproot, hypocotyl, and midstem of the resistant cultivars, the production of EPS I stopped. In the susceptible cultivar, however, EPS I production continued at the same apparent rate until at least day 8. EPS I production was also compared on a per bacterial cell basis. Surprisingly, the bacteria in the resistant cultivars produced, on average, approximately 15-fold more EPS I per cell than did the bacteria in the susceptible cultivar (12.5 pg of EPS I per bacterium in the resistant cultivars versus 0.7 pg of EPS I per bacterium in the susceptible cultivar).

Bacterial ingress into unwounded roots after soil infestation.

One possible explanation for the lower number of *R. solanacearum* cells in cvs. L285 and Hawaii 7996 is the reduced bacterial invasion of their roots. Therefore, the ability of bacteria to enter the taproot was determined for all cultivars. In two separate experiments, a suspension of *R. solanacearum* was poured onto the soil of unwounded plants. After 5 h, all of the bacteria on the exterior of the root system were removed or killed by washing the roots extensively with water until all of the soil was removed and then submerging them in 1% sodium hypochlorite for 1 min. After rinsing, the roots were sprayed with 80% ethanol, which was then burned off. When the taproots were quantitatively assayed for bacteria, >fourfold more bacteria were recovered from the taproots of cv. Marion (1.5×10^5 b/gpt) than from cv. L285 (3.6×10^4 b/gpt), and >13-fold more than from cv. Hawaii 7996 (1.1×10^4 b/gpt). Thus, the roots of susceptible plants were penetrated by *R. solanacearum* to a much greater extent than those of the resistant plants, suggesting that restriction of bacterial entry into the taproot may be an important factor in resistance to wilt disease.

Bacterial multiplication in plants after petiole inoculation.

Another possible explanation for the lack of wilt symptoms in the resistant cultivars after soil infestation was that the population of bacteria in the stem never reached levels required to block the flow of xylem fluid. Grimault and Prior (18) suggested that bacterial population densities in the midstem correlated with wilt symptoms in different cultivars of tomato. In our soil infestation experiments, the susceptible cultivar initiated wilt symptoms after bacterial populations exceeded approximately 4×10^7 b/gpt in the midstem.

To generate bacterial populations of this density in the midstems of the resistant cultivars, we inoculated 5×10^7 bacteria into the midstems via a cut petiole. Again, we observed that bacteria in the resistant cultivars multiplied and spread slower than those in the susceptible cultivars (Fig. 3). At the inoculation site on day 8, bac-

terial populations were approximately 10-fold greater in cv. Marion than in cv. L285, and almost 100-fold greater than in cv. Hawaii 7996 (Fig. 3B). Greater differences in bacterial populations were observed above and below the site of inoculation (Fig. 3A and C), with the greatest difference seen at the site 10 cm above. At this site, bacterial densities on day 8 were approximately 10-fold greater in cv. Marion than in cv. L285 and 1,000-fold greater than in cv. Hawaii 7996. Population densities in the midstems of all cultivars exceeded the hypothetical wilt threshold by approximately 10-fold, reaching at least 3×10^8 in the midstems of all cultivars; yet only cv. Marion exhibited wilt symptoms. Thus, populations exceeding the apparent wilt threshold in the midstems were correlated with wilt symptoms, but alone were not sufficient to induce them.

Bacterial multiplication in cv. Hawaii 7996 was sufficiently low that it was unclear if the bacteria could obtain the nutrients required for multiplication from the plant. To examine this more carefully, very low numbers (approximately 1,000) of *R. solanacearum* cells were petiole-inoculated into all three cultivars and multiplication was monitored. As a control, we also inoculated plants with *R. solanacearum* *hrpB* mutants, which are hindered in the ability to multiply in planta (4). Finally, sterile distilled water was inoculated with the same amount of each strain and incubated under similar conditions to determine how many cell divisions could result in the absence of additional nutrients. After 9 days, we found that the number of *R. solanacearum* cells doubled 25 times in cv. Marion, 15 times in cv. L285, and 10 times in cv. Hawaii 7996. The *R. solanacearum* *hrpB* mutant doubled three times in all cultivars, whereas the bacteria in the distilled water doubled four times. These results suggest that *R. solanacearum* cells were able to obtain, at least initially, sufficient nutrients for growth from the resistant plants, but that their multiplication was limited by an unknown factor.

To determine if a growth-inhibiting factor or nutritional deficiency of the xylem fluids of the resistant cultivars was responsible for reduced bacterial growth, we inoculated approximately 1,000 *R. solanacearum* cells into xylem fluids obtained from both infected and uninfected plants of each cultivar and used them as an in vitro growth medium. No differences in growth yields of *R. solanacearum* grown on xylem fluids from infected or uninfected plants of susceptible or resistant cultivars was observed, indicating that antibacterial substances or nutritional deficiencies in the xylem fluids of the resistant cultivars were not responsible for the reduced bacterial growth.

Localization of bacteria and EPS I in plants by immunofluorescence microscopy after soil infestation. To gain further insight into the differences in *R. solanacearum* colonization and EPS I production in the resistant and susceptible cultivars, im-

Fig. 3. Bacterial multiplication in petiole-inoculated tomato plants. Three tomato plants of the susceptible cv. Marion (filled circles) and the resistant cvs. Hawaii 7996 (open squares) and L285 (open circles) were inoculated with 5×10^7 bacteria via a cut petiole. Plants were harvested and enumerated for bacteria at **A**, 10 cm above the site of inoculation; **B**, the site of inoculation; and **C**, 10 cm below the site of inoculation.

munofluorescence microscopy was performed using antibodies directed against EPS I or β -galactosidase (which specifically labeled *R. solanacearum* cells because they had been engineered to constitutively express *lacZ* [35]). By day 5, bacteria colonized the taproot interior of cv. Marion and had produced EPS I (Fig. 4A). Similar results were observed with cv. L285 (data not shown); however, very few bacteria were observed in cv. Hawaii 7996 and they appeared to be contained in isolated microcolonies (Fig. 4B). In the hypocotyl of cv. Marion, bacteria colonized a majority of the xylem vessel elements (Fig. 5A). In cv. L285, bacteria were also observed in the xylem vessels, but to a lesser degree (data not shown). In contrast, in cv. Hawaii 7996, very few vessels were colonized and then with only low populations of cells (Fig. 5B). In cv. Marion, colonization of the intercellular spaces of the pith was regularly observed (Fig. 6A), but in cv. L285, this was rarely observed and, in cv. Hawaii 7996, this was never observed (Fig. 6B). In the petioles of cv. Marion, large numbers of bacteria colonized the xylem, the surrounding intercellular spaces, and parenchyma cells (Fig. 7A), producing copious amounts of EPS I (Fig. 7B). Fewer bacteria were observed in the petioles of cv. L285 than of cv. Marion and they remained associated with the xylem. In cv. Hawaii 7996, it was difficult to find any bacteria in the petioles (data not shown). In the midstem of cv. Marion, heavy colonization of the vascular tissue and the intercellular spaces surrounding the vascular tissue was apparent. Colonization of the midstem in cv. L285 was lower and restricted to the vascular tissue; in cv. Hawaii 7996, bacteria were sparse and restricted to the vascular tissue. We observed that bacteria were able to multiply and produce EPS I in both resistant and susceptible cultivars; however, in the resistant cultivars, the bacteria were restricted to the vascular tissues and the amount of EPS I per gram of plant tissue was reduced.

DISCUSSION

The ability to invade roots, establish an infection, colonize the plant, and produce virulence factors is necessary for *R. solanacearum* to cause disease in susceptible hosts (17,18,40). In this study, we found significant differences in the way *R. solanacearum* invaded the unwounded roots of susceptible and resistant tomato cultivars. The susceptible cv. Marion allowed rapid ingress of *R. solanacearum* into the taproot, which is not surprising given that previous studies showed that even *E. coli* could rapidly penetrate its taproot (35). In the resistant cvs. L285 and Hawaii 7996, ingress by *R. solanacearum* was 5- and 15-fold slower, respectively. These data indicate that resistance to wilt disease is correlated with resistance to ingress. Our results conflict with those of Grimault and Prior (17), who reported that resistance to root invasion did not correlate with resistance to wilt. However, in their study, the roots were wounded before inoculation, whereas, in ours, the roots were intact; this may explain the discrepancy. The importance of resistance to taproot invasion in the field is unknown, but it has the potential to contribute to the overall resistance to wilt disease.

Once bacteria invaded the roots, we observed differences in the way they spread to the hypocotyl and lowest petioles of the susceptible and resistant cultivars. For example, in the susceptible cultivar, bacteria spread to the midstem region by day 2, but were not detected in the resistant cultivars until day 5 (onset of wilt in the susceptible cultivar). These data are similar to those of previous studies that correlated rapid spread to the midstem with susceptibility (15,17,33). Previous studies also suggested that the initial spread is probably via the xylem vessel elements (16,40). However, the large population of bacteria observed in the continuum of intercellular spaces of the pith suggests that dissemination in the

Fig. 4. Immunofluorescence microscopy of bacteria and EPS I in taproots. **A**, Cross section of the primary root of the susceptible cv. Marion labeled with anti-EPS I antibody showing EPS I widely distributed in the vascular cylinder (arrowheads). **B**, Cross section of the primary root of the resistant cv. Hawaii 7996 labeled with anti- β -galactosidase antibody showing an isolated bacterial "microcolony" (arrows) within the vascular cylinder. Bars = 20 μ m.

Fig. 6. Immunofluorescence microscopy of EPS I in the intercellular spaces of the pith. **A**, Cross section of the hypocotyl in the susceptible cv. Marion labeled with anti-EPS I antibody showing the intercellular spaces of the pith containing EPS I (arrowheads). **B**, Cross section of the hypocotyl of the resistant cv. Hawaii 7996 showing the intercellular spaces of the pith with no infection. Bars = 20 μ m.

Fig. 5. Immunofluorescence microscopy of bacteria in the vascular tissues. **A**, Cross section of the hypocotyl region in the susceptible cv. Marion labeled with anti- β -galactosidase antibody showing a vascular bundle with bacteria (arrows) inside the xylem and surrounding cells. **B**, Cross section at the hypocotyl region in the resistant cv. Hawaii 7996 labeled with anti- β -galactosidase antibody showing only limited infection of the xylem vessels. Bars = 20 μ m.

Fig. 7. Immunofluorescence microscopy of bacteria and EPS I in the petioles of the susceptible cv. Marion. **A**, Vascular bundle in the petiole of cv. Marion labeled with anti- β -galactosidase antibody showing bacteria (arrows) in the vessel elements and surrounding intercellular spaces. **B**, Same as **A** except labeled with anti-EPS I antibody showing EPS I (arrowheads) in the xylem vessels and surrounding intercellular spaces. Bars = 20 μ m.

susceptible cultivar may also be enhanced by travel through these intercellular spaces. Furthermore, the thread-like trails of bacteria observed in the pith are consistent with movement through these spaces. In contrast, bacteria colonizing the resistant cultivars were considerably more restricted to the vascular bundles.

Rapid in planta multiplication has been associated with the ability to cause wilt disease symptoms (31,44). Our data indicated that *R. solanacearum* could grow to the same extent in xylem fluids obtained from infected and uninfected plants of both the resistant and susceptible cultivars. Thus, the inability of *R. solanacearum* to multiply rapidly in resistant cultivars was probably not related to growth-inhibiting compounds or nutritional deficiencies in the xylem fluids. The inability to rapidly multiply and spread in the resistant cultivars resulted in lower bacterial populations throughout the plants, especially in the midstems. Grimault et al. (15) suggested that the inability to spread rapidly and attain high numbers in the midstem was associated with resistance. Furthermore, they suggested the existence of a bacterial concentration threshold that must be surpassed to induce wilt symptoms. However, others have proposed that the presence of large numbers of *R. solanacearum* in the water-conducting vessels of the stem was not the only factor involved in wilt (5,17). Interestingly, when plants were inoculated with large amounts of bacteria and their population densities in the midstems exceeded the apparent wilt threshold by approximately 10-fold, neither resistant cultivar exhibited wilt symptoms, whereas the susceptible cultivar rapidly wilted and died. Thus, high bacterial densities in the midstem alone were not sufficient to induce wilt symptoms. However, it should be noted that the overall pattern of colonization resulting from inoculating bacteria into the petiole may significantly differ from that of plants grown in infested soils.

In the susceptible cultivar, markedly more EPS I was produced per plant than that in the resistant cultivars; however, there were greater amounts of EPS I produced per bacterium in the resistant cultivars. The apparent "overproduction" of EPS I by the bacteria in the resistant cultivars may be explained by one of the features of the complex regulatory network that controls EPS I biosynthesis in *R. solanacearum* (7,13,23). Expression of the *eps* operon in vitro is cell-density dependent and is not initiated until bacterial populations exceeded 10^7 cells per ml (7). Since bacteria in the resistant cvs. L285 and Hawaii 7996 were more restricted (i.e., more bacteria per unit volume) than those in the susceptible cv. Marion, an earlier induction of EPS I biosynthesis may have occurred, resulting in greater production of EPS I on a per cell basis. Consistent with this hypothesis, bacteria grown in vitro, where they are spatially less restricted than in planta, produce 10-fold less EPS I per cell than those grown in cv. Marion.

Immunofluorescence microscopy revealed that the distribution of bacteria and EPS I within the resistant and susceptible cultivars was strikingly different. In cv. Marion, bacteria and EPS I were observed throughout the vascular tissues and intercellular spaces of the pith. This contrasts with cv. L285, in which bacteria and EPS I were observed to be primarily in the vascular tissues, and in cv. Hawaii 7996, in which they were exclusively limited to the xylem vessels and the immediately surrounding intercellular spaces. The inability of the bacteria to spread from the vascular tissues of the hypocotyl and midstem into the intercellular spaces of the pith could be due to host defenses such as gums and tyloses (16,40). Alternatively, the restriction may result from a reduced ability of the *R. solanacearum*-produced plant cell wall-degrading enzymes to disrupt the cell walls of the resistant cultivars. Studies with resistant potato cultivars showed that higher percentages of methylated and branched pectins correlated with resistance to spread by *Erwinia* in stem tissues (29,31).

Several studies have presented evidence that resistance to bacterial wilt disease in tomato is complex and involves genes scattered throughout the tomato genome (8,21). Although the products and functions of these genes are unknown, they appear to somehow pre-

vent bacteria from readily entering and spreading throughout the plant, since the major common trait of wilt-resistant tomato cultivars is the restriction of bacterial spread. Indeed, even when large amounts of bacteria were injected directly into the stems of resistant cultivars, the bacteria were limited in their ability to spread as compared with that in the susceptible cultivar. The inability of the bacteria to spread throughout the plant is likely a key factor in wilt resistance.

Finally, even tomato cultivars considered to be highly resistant can succumb to specific strains of *R. solanacearum*, depending on the climate and location (41). Thus, a simple, rapid, sensitive, and inexpensive screening method that could differentiate varying degrees of resistance among cultivars would be useful in finding the most suitable cultivar for a specific region. We have demonstrated that the amount of EPS I produced per gram of plant tissue correlates with the level of resistance to *R. solanacearum*. Therefore, the ELISA for EPS I would be very useful as a means of testing the level of resistance of tomato cultivars to *R. solanacearum* before planting.

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