Distribution and Multiplication of *Ralstonia solanacearum* (Synonym *Pseudomonas solanacearum*) in Tomato Plants of Resistant Rootstock Cultivar LS-89 and Susceptible Ponderosa

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Abstract

We examined the distribution and multiplication of *Ralstonia solanacearum* (Synonym *Pseudomonas solanacearum*) in roots and stems of tomato plants of resistant rootstock cultivar LS-89 and susceptible cultivar Ponderosa. In a growth chamber at 30°C, 30-day-old seedlings were inoculated with *R. solanacearum* strain 8107S after trimming lateral roots and immersing the root ball in a bacterial suspension of 10^7 cfu/ml. In inoculated Ponderosa plants, bacteria were recovered from all taproot and stem samples tested. Pathogen density increased in root and stem tissues to 10^8 or 10^9 cfu/g fresh matter in 10 days. Inoculated LS-89 plants were latently infected with the pathogen. Recovery of the pathogen in these LS-89 plants at 14 days after inoculation was about 90% in taproots and the upper hypocotyls, and about 80% in the first internodes, but the percentage decreased higher in the stem (30% recovery at the fifth internode). Pathogen density increased to 10^6 cfu/g fresh matter in taproots, upper hypocotyls and the first internodes in the first 4 days, but it was steady afterwards. Transverse sections of the upper hypocotyls of infected plants showed bacterial masses in the primary and secondary xylem tissues in Ponderosa, but only in a portion of the primary xylem tissues in LS-89. These results indicate that the bacteria are localized in the primary xylem tissues and their movement is limited in resistant LS-89 plants.

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Key words: *Ralstonia* (*Pseudomonas*) *solanacearum*, tomato, resistance, rootstock.

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* (Synonym *Pseudomonas solanacearum*²⁰⁾), distributed widely in tropical, subtropical and some warm temperate regions worldwide, is one of the most important diseases of solanaceous crop plants⁹⁾. As the most successful method of control for bacterial wilt is the use of resistant cultivars¹⁶⁾, several highly resistant tomato rootstocks have been developed in Japan^{12,21)}. Grafting of susceptible tomato cultivars onto resistant rootstocks has been adopted to manage bacterial wilt^{11,12,21)}.

In our previous report¹³⁾, plants of the resistant rootstock cultivar LS-89 (*Lycopersicon esculentum*) which were inoculated with *R. solanacearum* after trimming the lateral roots, and immersing the root ball in bacterial suspension, showed latent infection or mild wilting. When scions of the susceptible cultivar Ponderosa (*L. esculentum*) grafted on LS-89 rootstocks were similarly inoculated with the pathogen, many Ponderosa scions had infection with the pathogen moved from latently infected LS-89 rootstocks and wilted.

To clarify the mechanism of bacterial wilt resistance

in LS-89, this paper reports precisely on the distribution and density of *R. solanacearum* in root-inoculated plants of resistant LS-89 and susceptible Ponderosa and on the movement of the pathogen in their stems.

MATERIALS AND METHODS

Plants Resistant tomato LS-89²¹⁾ and susceptible Ponderosa were used. Tomato cultivars were individually seeded in peat moss pellets (Jiffy-9, 50 mm single, Jiffy Products Ltd.) in a greenhouse at $25 \pm 5^\circ\text{C}$. About 30 days after sowing, seedlings of LS-89 having five to six leaves and those of Ponderosa having four to five leaves were inoculated.

Bacteria *R. solanacearum* strain 8107S, which is a fluidal spontaneous mutant from the strain 8107¹³⁾ and resistant to 200 $\mu\text{g/ml}$ streptomycin sulfate, was used to permit sensitive counting of bacterial colonies on the selective agar medium described below⁷⁾. Antibiotic resistance of 8107S remained stable after its recovery from infected tomato plants. Pathogenicity of 8107S to tomatoes is essentially similar to 8107. Symptoms caused by 8107S in LS-89 is a little milder than those by 8107, but the density of 8107 and 8107S in latently infect-

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ed LS-89 tissues in our preliminary study did not differ. Cultures were stored in a solution of 100 g skimmed milk and 10 g Na-glutamate per liter at -20°C to avoid possible development of non-pathogenic mutants.

Inoculation The stock culture was thawed and streaked on the selective agar medium of Hara and Ono⁷⁾ containing 200 $\mu\text{g/ml}$ streptomycin sulfate. After incubation at 30°C for 48 hr, bacteria were collected using a plastic spatula and suspended in distilled water. The suspension was diluted further with water to have bacteria at 10^7 cfu/ml as estimated with a colorimeter ($OD_{660}=0.22: 5 \times 10^8$ cfu/ml, Spectronic 20, Bausch & Lomb). Tomato seedlings were inoculated by trimming lateral roots extending from peat moss pellets, then immersing the pellets in the bacterial suspension in a plastic container ($34 \times 24 \times 15$ cm) for 24 hr in a growth chambers under 30,000 lux light for 12 hr per day at constant temperature of 30°C . Sixty to 96 plants of each LS-89 and Ponderosa were used per treatment. The experiment was repeated eight times. Inoculated plants were grown in the growth chamber under the same conditions.

Recovery of bacterial pathogens from plant tissues Inoculated plants of LS-89 and Ponderosa were indexed daily for 14 days for disease severity following the numerical grading of Winstead and Kelman¹⁹⁾. Daily for the first 10 days and on the 14th day after the inoculation, plants were sampled randomly by dissecting

5-mm-long sections from taproots, upper hypocotyls (approximately 5 mm below the cotyledon) and the first, third and fifth internodes of seedlings. Sections were separately weighed and macerated with 1 ml of sterile distilled water using aluminum sticks in a test tube (1.5 cm in diameter and 10.5 cm long). After incubation of the samples in the test tube at 10°C for 12 hr to allow bacteria to ooze and disperse in the water, 100 μl of the tissue suspension were diluted serially and plated onto the selective agar medium⁷⁾ amended with the antibiotic. The plates were cultured at 30°C for 48 hr. Seedlings of the two cultivars mock-inoculated with distilled water served as the control. Colony number was counted and converted to indicate bacterial density in tissues in cfu/g of fresh matter (cfu/g FM).

Statistical analysis The Scheffé's F-test and the Fisher's exact probability test were used in analysis on pathogen density and pathogen recovery, respectively.

Light microscopy Fourteen days after inoculation, latently infected LS-89 plants and totally wilted Ponderosa plants were selected. A 5-mm-long section was sampled from the upper hypocotyl of each plant and fixed in a mixture of formalin, acetic acid and 50% ethanol (1 : 1 : 18 in volume) for 48 hr or longer. The fixed section was dehydrated through an ethanol and 1-butanol series and embedded in paraffin. Transverse sections, 15–20 μm in thickness, were obtained from embedded sections mounted on a rotary microtome and

Fig. 1. Percentage of wilting, percentage of recovery of the pathogen from upper hypocotyls, and disease severity index of seedlings of LS-89 and Ponderosa from 1 to 14 days after inoculation with *Ralstonia* (*Pseudomonas*) *solanacearum* 8107S. Percentage of wilting and bacterial recovery in upper hypocotyls and disease index were monitored on 9–12 randomly selected plants. Average of five replications.

stained with thionin and orange G¹⁵). Accordingly, bacterial masses were stained purple, lignified cells blue and parenchymatous cells yellow to orange. Thin sections were observed under a light microscope to locate bacterial masses in the stem tissues.

RESULTS

Recovery of the pathogen and conditions of inoculated plants

In Ponderosa, the pathogen was recovered from plants by 1 day after inoculation. Leaf wilting had begun by 5 days after inoculation (Fig. 1). By 7 days after inoculation, the percentage of pathogen recovery and the percentage of wilting in inoculated plants increased over time and had reached nearly 100% by 7 and 14 days after inoculation, respectively. By 14 days after inoculation, almost all inoculated plants showed severe wilting or death. In LS-89, the increase in the percentage of pathogen recovery was similar to that in Ponderosa, although none of inoculated plants showed wilting.

Distribution and density of the pathogen in plants of LS-89 and Ponderosa

In inoculated LS-89 plants, pathogen density increased to 10^6 cfu/g FM in taproots, the upper hypocotyls and the first internodes in the first 4 days after inoculation, then steadied (Fig. 2). The density in the third internode remained at a low level even at 10 days after inoculation ($p < 0.05$). However, in inoculated Ponderosa plants, pathogen density in all the parts increased with time after inoculation and reached 10^8 or 10^9 cfu/g FM by 10 days (Fig. 2). At 10 days after inoculation, the density was significantly higher in all parts of Ponderosa than in LS-89 ($p < 0.05$).

At 14 days after inoculation, bacteria were recovered

Fig. 3. Recovery of the pathogen from taproots and stems at the basal part to upper internodes of LS-89 and Ponderosa at 14 days after inoculation with *Ralstonia (Pseudomonas) solanacearum* 8107S. Bacterial recovery was monitored on 9–12 randomly selected plants. Average of five replications.

from all taproot and stem sections tested in Ponderosa (Fig. 3). In LS-89, recovery was about 90% in taproots and upper hypocotyls, but gradually decreased in the upper internodes to reach 30% in the fifth internodes ($p < 0.05$).

Distribution of the pathogen in stem tissues

Cut surfaces of traverse sections from the upper

Fig. 2. Development of bacterial density in taproots and stems at the basal part to upper internodes of LS-89 and Ponderosa after inoculation with *Ralstonia (Pseudomonas) solanacearum* 8107S. Bacterial density was monitored on 5–7 randomly selected plants. Average of five replications.

hypocotyl by light microscopy showed brown discoloration in xylem tissues in Ponderosa but no such discoloration except occasional brown spots in LS-89 (data not shown). In infected Ponderosa plant tissues, purple-stained bacterial masses occurred in the primary and secondary xylem vessels (Plate I-2), occupying a major space in each vessel (Plate I-2). Often, bacterial masses were observed in intercellular spaces of necrotic cells in xylem and nearby pith tissues (data not shown). Occasionally, large, bacteria-filled cavities due to cell necrosis occurred in these tissues. In Ponderosa, tylose formation was observed rarely in primary and secondary xylem vessels in inoculated (*e.g.*, Plate I-2) and mock-inoculated plants (*e.g.*, Plate I-1). No purple-stained bodies were observed in the vessels of mock-inoculated Ponderosa plant tissues (Plate I-1).

In infected LS-89 plant tissues, bacterial masses occurred in the primary xylem tissues but not in secondary xylem tissues (Plate I-4, 6). In tissues of upper hypocotyls with brown spots in the cut surface, bacterial masses were observed in primary xylem vessels and occasionally in surrounding necrotic cells (Plate I-6). Tissues of upper hypocotyls that showed no brown spots in the cut surface had bacterial masses in necrotic cells or intercellular spaces (Plate I-5). Generally, the size of bacterial masses in each xylem vessel was smaller in LS-89 than in Ponderosa (Plate I-2 and I-4). In infected LS-89, marked histological changes were not observed either in xylem or other tissues except for the bacterial masses in cells or intercellular spaces (compare Plate I-3 and I-6). In LS-89, tyloses formed in some vessels of the primary and secondary xylems, both in inoculated and mock-inoculated plants (data not shown). No bacterial masses were observed in xylem tissues in mock-inoculated plant tissues (Plate I-3).

DISCUSSION

After inoculation with *R. solanacearum* 8107S by the immersion of cut roots in a bacterial suspension of 10^7 cfu/ml and growth chamber for 14 days at 30°C, almost all LS-89 plants had latent infection and all of inoculated Ponderosa plants had wilted. LS-89 plants showed no retardation in plant growth and no sign of abnormality in the level of respiration of plant tissues ($\text{g H}_2\text{O h}^{-1} \text{ plant}^{-1}$) (data not shown). Pathogen density increased in infected taproots and stems of both cultivars, but to a lesser extent in LS-89 than in Ponderosa. The density was especially low in upper internodes in LS-89. In addition, the recovery of bacteria from latently infected LS-89 tissues was higher in the basal part of the stems than in the upper parts. These results, indicating that bacterial movement and multiplication is limited in stems of LS-89, agree with the results of Grimault and Prior⁴⁾. Limitation in movement and/or multiplication of bacterial wilt pathogens in plants has also been reported in resistant cultivars in other solanaceous crops, including potato¹⁾, tobacco¹⁴⁾, eggplant and

pepper⁵⁾.

In our previous report on tomato¹³⁾, *R. solanacearum* moved from inoculated resistant LS-89 rootstocks into the susceptible Ponderosa scions and caused wilting. Wilting due to bacteria in susceptible Ponderosa scions grafted on LS-89 was milder than that in Ponderosa or LS-89 scions grafted on Ponderosa. Because of the limitation in both bacterial movement and multiplication in LS-89 rootstocks, the movement of bacteria from rootstocks to scions may be less in number when LS-89 was used as the rootstock than when Ponderosa was used.

In this study, light microscopy of tissues samples collected from upper hypocotyls showed bacterial masses only in the primary xylem in LS-89, but in both the primary and secondary xylems in Ponderosa. Furthermore, the frequency of bacterial masses in vessels in transverse sections was high in Ponderosa, but low in LS-89. In tomato seedlings in which the secondary xylem had not been developed yet, bacterial colonization was observed in some vessels of the primary xylem in hypocotyls of partially wilted plants¹⁷⁾ and in the protoxylem and metaxylem (mainly in pitted vessels (metaxylem vessels)²⁾) in epicotyls of susceptible plants, as opposed to primarily in the protoxylem vessels in epicotyls of resistant plants⁶⁾. The results in this study and earlier studies^{6,13,17)} indicate that *R. solanacearum* colonizes vessels of the protoxylem and primary and secondary xylem in susceptible cultivars, whereas it colonizes inner parts of the xylem, mainly the primary xylem, in resistant cultivars. The spread of the pathogen horizontally from the protoxylem or primary xylem tissues to other xylem tissues seems to be limited in resistant cultivars.

Wilting of plants infected with bacteria is likely induced by the blocking of water conductance resulting from mechanical plugging of xylem vessels³⁾, as has also been described in bacterial wilt of tomato with vessels becoming plugged by bacterial masses¹⁰⁾, extracellular polysaccharides⁹⁾ or tyloses¹⁸⁾. *R. solanacearum*-infected LS-89 escaped from wilting because bacterial masses were low in frequency and smaller in size in stem tissues.

Tylose formation in infected Ponderosa and LS-89 plants was essentially similar, inconsistent with the report of Grimault *et al.*⁶⁾ correlating a limitation of bacterial spread in a resistant tomato with the production of tyloses in bacterial colonized and adjacent vessels. Tylose formation may not be a necessary factor to limit bacterial movement. Why *R. solanacearum* does not multiply or migrate extensively in tissues of resistant plants remains to be clarified.

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和 文 摘 要

中保一浩：抵抗性トマト台木品種 LS-89 と感受性品種ボンデローザの植物体内での青枯病菌の分布と増殖

青枯病抵抗性トマト台木品種 LS-89 と感受性品種ボンデローザの植物体内における青枯病菌の分布と増殖を調べた。30°C のグロースチャンバー内で断根した 30 日苗を青枯病菌 8107 S 懸濁液 (10^7 cfu/ml) に浸漬し接種した。ボンデローザでは接種 14 日目のすべての主根、茎部の試料から病原細菌が検出された。根部、茎部の青枯病菌密度は接種 10 日目には生重量 1 g 当たり 10^8 あるいは 10^9 個に増加した。一方、接種した LS-89 は無病徴感染し、接種 14 日目の主根と胚軸上部での青枯病菌の検出率は 90% 以上であった。茎からの検出率は第 1-2 葉間の茎部で約 80% であり上位の茎部にいくに従い低下した (第 5-6 葉間の茎部で 30%)。青枯病菌密度は接種 4 日目に主根、胚軸上部および第 1-2 葉間の茎部において生重量 1 g 当たり 10^6 個まで増殖したが、その後は一定であった。胚軸上部の横断面の顕微鏡観察により、青枯病菌はボンデローザの一次および二次木部組織に広く認められたが、LS-89 では一次木部組織の一部のみに分布していた。以上の結果、抵抗性 LS-89 では青枯病菌の一次木部組織での局在化と植物体内での移行抑制が認められた。

Plate I

Plate I Transverse sections of the upper hypocotyls (approximately 5 mm below the cotyledon) in Ponderosa and LS-89 plants inoculated or mock-inoculated with *Ralstonia (Pseudomonas) solanacearum* 8107S.

1. Mock-inoculated Ponderosa plant. Bar : 100 μ m.
2. Wilted Ponderosa plant with bacterial masses (arrowheads) in primary xylem and secondary xylem vessels. Bar : 100 μ m.
3. Mock-inoculated LS-89 plant. Bar : 100 μ m.
4. Inoculated LS-89 plant with bacterial masses (arrowheads) in the primary xylem. Bar : 100 μ m.
5. Primary xylem in the upper hypocotyl of LS-89 with bacterial masses (arrowheads) in necrotic cells or intercellular spaces. Bar : 100 μ m.
6. Inoculated LS-89 plant with brown spots in cut surface has bacterial masses (arrowheads) in its primary xylem. Bar : 100 μ m.