The Behavior of *Lux*-marked *Ralstonia solanacearum* in Grafted Tomato Cultivars Resistant or Susceptible to Bacterial Wilt

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Abstract

Ralstonia solanacearum OE1-1 was transformed with pNP126 carrying a *luxCDABE* operon of *Vibrio fischeri* and a promoter region derived from the genomic DNA of *Burkholderia glumae*. Intensity of bioluminescence from YN5 thus obtained positively correlated with growth of the bacteria from the lag phase to the stationary phase *in vitro*. In this study, we used the VIM camera equipped with the ARGUS 50 to successively observe bioluminescence and the development of bacterial wilt in singly and doubly grafted tomato plants in various combinations of the resistant LS-89 and the susceptible Oogata-Fukuju. The degree of bacterial growth in the roots and collars was one of the determinants for the induction of bacterial wilt in tomato plants. Resistance of LS-89 resulted from suppression of bacterial proliferation in the roots and stems below the first leaf. In grafted tomato plants using LS-89 as the rootstock, all rootstocks were latently infected with the bacteria. The bacteria were also recovered from susceptible scions even though the plants did not wilt. In some plants, the bacteria proliferated in the susceptible scions. These plants wilted heavily. Taken together, suppression of bacterial proliferation in the roots and the stems below the first leaf of resistant rootstocks affected the bacterial growth in the susceptible scions and quantitative control of resistance against development of the disease.

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Key words : *Ralstonia solanacearum*, bacterial wilt of tomato, grafted tomato plants, LS-89.

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* (synonym *Pseudomonas solanacearum*) (Smith) Smith, is one of the most devastating bacterial plant diseases of tomato in the tropics, subtropics, and warm temperature regions worldwide⁸). The bacterium invades plant vascular tissues from wounded roots or natural openings from which secondary roots subsequently emerge^{11,21}). Bacterial colonization in the stems results in browning of the xylem, foliar epinasty and a lethal, generalized wilt¹).

Control of the disease with chemicals is mostly ineffective or is not adapted to low-income farming systems⁹). For the control of the disease in Japan, the soil must be sterilized by solarization or with chemicals such as methyl bromide or chlorpicrin, either independently or in combination¹⁴). However, soil sterilization does not completely control the disease because chemicals are absorbed by soil particles and degraded by microorganisms.

The use of resistant varieties of tomato is the most

effective means for the control of the disease, but no fresh market varieties of tomato possess both high resistance and high fruit quality. So grafting tomato cultivars susceptible to bacterial wilt onto resistant rootstocks is a means to control the disease^{1,14,18,24,25}). However, susceptible scions grafted onto resistant rootstocks often wilt in fields^{5,8,14,16}). The degree of disease resistance is inversely related to the spread of *R. solanacearum* in the stem tissues^{4,19}), which is limited in the vascular tissues of resistant tomato cultivars. Nakaho¹⁵) has further suggested that the horizontal spread of pathogen from the protoxylem or primary xylem tissues to other xylem tissues is limited in resistant cultivars.

The *lux* genes have been successfully expressed in diverse bacteria ; thus, the bioluminescence detection technique would have broad applicability in the study of the behavior of both rhizosphere and epiphytic bacteria^{9,10,20,22}). Luminescence-marker systems have now been used for environmental detection of a wide range of microorganism in soil and plants. Only living bacterial cells with introduced *lux* genes can produce

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bioluminescence, because the production of the substrate for the enzymatic reaction requires active electron transfer²⁰.

Luciferase and reductase are the key enzymes required for bioluminescence. The intensity of bioluminescence is strongly dependent on the cellular level of ATP to recycle tetradecanal, the luciferin for light production. However, in previous studies using X-ray films and the CCD camera, obtaining images of bacterial bioluminescence in a short time and measuring bacterial density quantitatively was difficult. The use of a video-intensified microscopy (VIM) camera results in detecting single photons in a short time, and excitation light is unnecessary to observe the bioluminescence^{7,9,10,12,23}.

We previously observed the growth and movement of *P. cichorii* transformed with pNP126 containing a *luxCDABE* operon and a promoter region derived from genomic DNA of *Burkholderia glumae* in lettuce leaf with the VIM camera equipped with the ARGUS 50¹⁰. We found that bacterial rot followed bacterial multiplication in the intercellular spaces of the epidermis and mesophyll and bacterial movement through the vascular bundle in the lettuce leaf. Disease development also required *de novo* protein synthesis in the tissues.

In this study, we used a VIM camera equipped with the ARGUS 50 to successively observe bioluminescence over time along with the development of bacterial wilt in singly grafted and doubly grafted tomato plants in various combinations of the resistant LS-89 and the susceptible Oogata-Fukuju. We also discuss the relationship between bacterial proliferation and disease development in tomato plants.

MATERIALS AND METHODS

Plants Seeds of a susceptible tomato (*Lycopersicon esculentum*) cultivar, Oogata-Fukuju (OF), and a resistant tomato cultivar, LS-89 (LS), a selection from Hawaii 7998²⁸, were purchased from Takii & Co., Ltd. Tomato seedlings were grown in soil (Compal, Sumitomo Forestry) in a nursery box (550 mm × 300 mm, 50 holes/box) in a greenhouse under 25 ± 5°C.

Fig. 1. Single and double grafts of tomato plants using tomato cultivars Oogata-Fukuju and LS-89 susceptible and resistant to bacterial wilt, respectively. Arrows show the positions of grafting.

Chisel-type approach grafts were carried out on 5-week-old tomato plants at the two- or three-leaf stage. Rootstocks were topped 10 mm below their cotyledon, and the cuttings were used as scions (Fig. 1). Scion/rootstock combinations of singly grafted plants were LS/OF, OF/LS, OF/OF and LS/LS. For double grafts, the second of which was carried out 1 week after the first, the middle scion contained their cotyledon. Apical scions were approximately 30 mm in length and included two or three leaves. Apical scion/middle scion/rootstock combinations of doubly grafted plants were LS/LS/LS, OF/OF/OF, OF/LS/LS, LS/OF/LS, LS/OF/OF and OF/LS/OF. The junctions between graft and receptor were supported by a plastic clip (Superwith, Sakata). The grafted tomato plants were placed in an incubator at 25°C with 100% relative humidity for 4 days and then in a shaded room for 3 days.

Bioluminescent *R. solanacearum* YN5 *R. solanacearum* OE1-1²⁹, a gift from Dr. H. Date, was triparentally transformed with plasmid pNP126 (Fig. 2)^{9,10} via an intermediate host *Escherichia coli* HB101 harboring a helper plasmid pRK2013 for transconjugation. The triparental mating was performed by mixing cells in the late exponential phase¹³. *R. solanacearum* transconjugants were screened on the selective agar media of Hara and Ono⁶ containing kanamycin and tetracycline at 50 µg/ml and 20 µg/ml, respectively. Kanamycin- and tetracycline-resistant clones were incubated overnight in PS media²⁷ containing kanamycin at 50 µg/ml, diluted to 1.0 × 10⁸ cfu/ml photometrically (1.0 × 10⁸ cfu/ml had an optical density of 0.1 at 600 nm) with the PS media containing kanamycin at 50 µg/ml, then incubated at 25°C for 1 hr. Bioluminescence of each bacterial suspension was measured with a luminometer (Luminescence Reader, ALOKA).

Inoculation A single colony of the fluidal type *R.*

Fig. 2. Plasmid vector pNP126. pro, a promoter region derived from *Burkholderia glumae*; lux, *lux* operon derived from *Vibrio fischeri*; pBR ori, ori derived from pBR322; TcR, tetracycline-resistant gene; NeoR, neomycin-resistant gene.

solanacearum transformants was grown at 30°C for 48 hr on the selective agar medium of Hara and Ono containing kanamycin and tetracycline at 50 µg/ml and 20 µg/ml, respectively. The inoculum was incubated in PS-medium containing kanamycin at 50 µg/ml, and the bacterial suspension was spectrophotometrically adjusted to 1.0×10^7 cfu/ml. Roots of 25 tomato plants were dipped into the bacterial solution for 30 min, then washed in flowing water for 1 min. After inoculation, tomato plants were grown in water culture pots (diameter, 65 mm; height, 65 mm; Yamato Water Culture Pot No. 1, Yamato Plastic Co. Ltd.) in five times-diluted Hoagland's solution at 25°C in a growth chamber under 20,000 lux for 12 hr per day. Inoculation test had five replicates.

Bioluminescence in planta Bioluminescence was observed in all plants inoculated with YN5. Images of bioluminescence were obtained by focusing the VIM camera equipped with the ARGUS 50 (Hamamatsu Photonics) on the sample and exposing for 1 min⁹⁾. Images were superimposed on those obtained with incident light. Images were directly recorded in TIFF format at a PC workstation, for processing with Adobe Photoshop 4.0 and a Macintosh.

Bacterial population in tomato plants Roots of singly grafted OF/OE, LS/LS, LS/OE and OE/LS plants were dipped into *R. solanacearum* YN5 at 1.0×10^7 cfu/ml. The middle and top of stems and collars were cut from five of each grafted plants 2, 6 and 10 days after inoculation (DAI) and macerated in a test tube (15 mm in diameter and 180 mm long) using an aluminum stick with 2 ml sterile distilled water. The test tube was then incubated at 9°C for 16 hr. The original solution and its 10-fold serial dilutions were dispensed in 0.1 ml samples and spread onto three plates of the selective agar

media of Hara and Ono and the media containing kanamycin at 50 µg/ml. The number of colonies was counted 4 days after incubation at 30°C to estimate the population.

RESULTS

Bioluminescent *R. solanacearum* YN5

R. solanacearum transformants with pNP126 were screened for bioluminescence, plasmid maintenance and pathogenicity to OE plants. The intensity of bioluminescence of a transformant, YN5, in PS-media was 500 times as high as *E. coli* HB101 transformants carrying pNP126, and pathogenicity of the transformant was comparable to that of the parental strain *R. solanacearum* OE1-1. The transformant had high plasmid maintenance in antibiotics-free PS medium and *in planta*. Therefore, we used YN5 in further studies.

In the PS medium, the intensity of bioluminescence derived from YN5 increased from the lag phase to the stationary phase of bacterial growth (Fig. 3).

The percentage of wilting plants

OE plants inoculated with YN5 started to wilt 3 DAI, and all plants were severely wilted by 7 DAI. The percentage of wilting LS plants 15 DAI was 13.6% (Table 1).

Grafted plants in combinations of OE/OE, LS/OE, OE/OE/OE, LS/OE/OE and OE/LS/OE were heavily wilted, and the percentage of wilting plants at 15 DAI was above 80 (Table 1). About 90% of LS/LS, LS/LS/LS and OE/LS/LS plants did not wilt, while about 50% of OE/LS and LS/OE/LS plants wilted.

Fig. 3. Growth of *Ralstonia solanacearum* YN5 and the bioluminescence derived from the bacteria in a PS medium. Bacterial bioluminescence was measured with a luminescence leader.

Table 1. The percentage of wilting plants in singly grafted and doubly grafted tomato plants in various combinations of the susceptible Oogata-Fukuju and the resistant LS-89 after inoculation with *Ralstonia solanacearum* YN5^{a)}

Plants ^{b)}	% wilting plants ^{c)}
Oogata-Fukuju (OE)	98.1A ^{d)}
LS-89 (LS)	13.6D
OE/OE	100 A
LS/OE	82.0B
LS/LS	11.1D
OE/LS	52.2C
OE/OE/OE	100 A
LS/OE/OE	92.6A
OE/LS/OE	93.9A
OE/LS/LS	7.7D
LS/OE/LS	50.0C
LS/LS/LS	10.2D

a) Roots of plants were dipped into *R. solanacearum* YN5. For details, refer to the text.

b) Scion/rootstock or apical scion/middle scion/rootstock.

c) Assessed 15 days after inoculation.

d) D.M.R.T. ($p=0.05$)

Behavior of bioluminescent *R. solanacearum* YN5 in planta

Bioluminescence in the susceptible OF plants was first observed in the roots and collars 1 DAI (Fig. 4-1). The intensity of bacterial bioluminescence in the collar reached a maximum 7-8 DAI and thereafter decreased. In the middle and the top of the stem, bioluminescence was first observed 4 DAI and reached a maximum 8 DAI. Thereafter, it decreased and was not observed at all at 15 DAI. On the other hand, bioluminescence in the resistant LS plants was only observed in the roots and collars (Fig. 4-1).

In singly grafted OF/OF and LS/LS plants, bacterial bioluminescence was similar to that in OF plants and LS plants, respectively (Fig. 4-2). In LS/OF plants, bioluminescence in the rootstocks was first observed 1 DAI and reached a maximum by 7-8 DAI. Bioluminescence was then observed in the scions, and the plants wilted heavily. In OF/LS plants, bioluminescence in the rootstocks remained weak. Then the plants in which bioluminescence had reached a maximum in the scions 7-8 DAI wilted heavily. On the other hand, the plants in which bioluminescence was not observed in the scions did not wilt.

In doubly grafted OF/OF/OF and LS/OF/OF plants with the susceptible OF used as the middle scions and the rootstocks, bioluminescence was strong in the rootstocks and then in the middle scions, and the plants wilted heavily (Fig. 4). In LS/LS/LS and OF/LS/LS plants with the resistant LS used as the middle scions and the rootstocks, weak bioluminescence was observed only in the roots and collars similar to that in the resistant LS plants. In OF/LS/OF plants, bioluminescence was strong in the rootstocks but weak in the middle scions, and then was strong in the apical scions, and the plants wilted heavily. In LS/OF/LS plants, weak bioluminescence was first observed in the rootstocks, and the plants with strong bioluminescence in the middle scion wilted heavily.

Bacterial population in tomato plants

The population of kanamycin-resistant *R. solanacearum* increased by an order of 3 to 4 in both the scions and the rootstocks of OF/OF plants and reached 2.5×10^8 and 1.4×10^9 cfu/g, respectively (Table 2). The bacterial population in LS/LS plants did not change much remaining at 10^3 - 10^4 cfu/g in the scion and 10^5 - 10^6 cfu/g in the rootstocks. The bacterial populations in LS/OF and OF/LS plants resembled those in the OF/OF and LS/LS plants, respectively. It should be noted that bacteria were present in the scions of non-wilting OF/LS plants at 10^3 to 10^4 cfu/g. The presence or absence of kanamycin did not affect the assessment of the bacterial populations (data not shown); bioluminescence was emitted from all colonies obtained on kanamycin-free medium and kanamycin-containing medium.

Fig. 4. Bioluminescence of *Ralstonia solanacearum* YN5 in tomato plants after inoculation with *R. solanacearum* YN5^a). Figures represent days after inoculation. For details, refer to the text. 1. Bioluminescence in tomato cultivars Oogata-Fukuju (OF) and LS-89 (LS). 2. Bioluminescence in grafted tomato plants in various scion/rootstock combinations of OF and LS. 3. Bioluminescence in double grafted tomato plants in various apical scion/middle scion/rootstock in combinations with OF and LS.

DISCUSSION

There are many reports on the behavior of *R. solanacearum* in host plants, but these previous results were obtained using methods which disrupted host tissues or depended on selective media. The disruption of plant tissues does not allow subsequent examination of the bacterial behavior and the disease development in the same plant tissues. Results from the present study using the VIM camera and the ARGUS 50 as a luminescence detection technique demonstrate that the relative intensity of bioluminescence derived from the genetically engineered bioluminescent bacteria YN5 was positive-

Table 2. Population of *Ralstonia solanacearum* YN5 in singly grafted tomato plants in various combinations with the susceptible Oogata-Fukuju (OF) and the resistant LS-89 (LS)

Plants ^{a)}	Symptom ^{b)}	Parts	Population of YN5 (cfu/g)		
			2 days ^{c)}	6 days	10 days
OF/OF	Wilting	Scion	$(2.6 \pm 1.6) \times 10^{4d)}$	$(2.5 \pm 0.21) \times 10^8$	$(7.5 \pm 3.0) \times 10^9$
		Rootstock	$(1.6 \pm 1.5) \times 10^5$	$(1.4 \pm 0.50) \times 10^9$	$(1.9 \pm 0.28) \times 10^9$
LS/LS	Non-wilting	Scion	$(2.5 \pm 1.3) \times 10^3$	$(2.6 \pm 0.85) \times 10^4$	$(1.0 \pm 0.071) \times 10^3$
		Rootstock	$(3.5 \pm 2.9) \times 10^5$	$(6.7 \pm 1.9) \times 10^5$	$(2.3 \pm 2.5) \times 10^6$
LS/OF	Wilting	Scion	$(3.5 \pm 1.9) \times 10^3$	$(7.7 \pm 0.49) \times 10^7$	$(8.9 \pm 1.6) \times 10^9$
		Rootstock	$(4.9 \pm 4.2) \times 10^5$	$(3.9 \pm 4.6) \times 10^9$	$(1.6 \pm 0.92) \times 10^9$
OF/LS	Non-wilting	Scion	$(6.0 \pm 1.4) \times 10^3$	$(2.1 \pm 0.14) \times 10^4$	$(1.2 \pm 0.49) \times 10^3$
		Rootstock	$(2.1 \pm 2.7) \times 10^5$	$(3.5 \pm 2.8) \times 10^6$	$(8.9 \pm 0.19) \times 10^5$
	Wilting	Scion	—	—	$(8.2 \pm 5.2) \times 10^9$
		Rootstock	—	—	$(6.0 \pm 2.0) \times 10^6$
		Scion	—	—	—
		Rootstock	—	—	—

a) Scion/rootstock.

b) Symptoms were observed 10 days after inoculation. For other conditions, refer to the text.

c) Days after inoculation with *R. solanacearum* YN5.d) Means $\pm$ SE of five replicates.

ly correlated with the growth of bacteria both on the PS medium from the lag to the stationary phase and within the tomato plants. Therefore, bioluminescent YN5 and this luminescence detection technique can be used to study the relationship of bacterial behavior and disease development of bacterial wilt in the same plant without destroying the samples.

The most widespread control strategy against bacterial wilt is the use of resistant cultivars^{1,14,18,24,25)}. Disease resistance of grafted plants is generally attributed to the absence of bacterial colonization in the resistant cultivars⁸⁾, but latent infections were demonstrated in bacterial wilt-resistant cultivars⁹⁾. Resistance to bacterial wilt is not associated with inhibition of bacterial root invasion but with the ability of the plant to limit bacterial colonization in the stem⁴⁾. Since bacterial colonization in the vascular tissues of the stem correlated with the frequency of bacterial wilt in the field, the level of resistance appears to correlate with the limitation of bacterial spread in plants^{4,19)}.

Grimault and Prior have reported the importance of bacterial latent infections in tomato xylem vessels⁵⁾. Vasse *et al.*²⁶⁾ have reported that after colonization in exudation sites such as root extremities and the axils of secondary roots, the bacteria intercellularly infect the inner cortex and the vascular parenchyma. Following infection, the bacteria invaded the protoxylem vessels. The results in this study show that tomato plants, either resistant LS or susceptible OF, in which the bacteria spread into the upper stems following a high proliferation of bacteria in the roots and the collars, would wilt. The root and the collars in non-wilting plants are latently infected with the bacteria.

Our previous studies revealed that the bacteria grow not only in the xylem vessels but also in the intercellular spaces and the inner spaces between the epidermis and the cortex in wilting tomato plants⁹⁾. In addition, a quantitative difference in bacterial bioluminescence between susceptible tomato cultivars and resistant ones

inoculated with YN5 could be more distinctly observed in the upper stems than in the collars of tomato plants¹⁷⁾. Bacterial proliferation in the roots and collars and the subsequent spread of the bacteria into the upper stems are crucial factors for the development of bacterial wilt in the tomato plants.

Grimault and Prior⁵⁾ have reported that resistant scions grafted onto susceptible rootstocks wilted and have suggested that vascular tissues of resistant cultivars are not as tolerant to higher bacterial populations as are susceptible ones and that resistance is correlated with the limitation of bacterial spread in the lower part of the stem. Nakaho *et al.*¹⁶⁾ suggested that bacterial wilt of susceptible scions grafted on resistant rootstocks is due to the movement of the bacteria from latently infected rootstocks into the scions. Multiple genes contribute to the resistance in LS to bacterial wilt. The resistance quantitatively controls disease development, depending on environment factors such as the pathogen density, cultivation condition and so on⁸⁾.

In the present study, the percentage of wilting OF/LS/LS plants was equal to that of wilting LS and LS/LS plants, and bacterial bioluminescence in OF/LS/LS plants was very similar to that in the LS and LS/LS plants.

Resistance of LS results from the suppression of bacterial proliferation in the roots and the stems below the first leaf. Moreover, in the susceptible scions of about 50% of the OF/LS and LS/OF/LS plants using LS as the rootstocks, the bacteria latently infected the rootstocks. Movement into the scions did not lead to a large increase in the bacterial population, and the grafted plants did not wilt. Taking these results together, suppression of the bacteria proliferation in the roots and the stems below the first leaf of resistant rootstocks affect bacterial growth in the susceptible scions and the quantitative control of resistance against development of the disease.

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和 文 摘 要

曳地康史・中澤（那須）佳子・北之園忍・鈴木一実・奥野哲郎：
青枯病抵抗性と感受性トマト品種による接木トマトにおける
lux 標識 *Ralstonia solanacearum* の挙動

Vibrio fischeri 由来の *lux* オペロンと *Burkholderia glumae* ゲノム DNA 由来のプロモーター領域を有する pNP126 で *Ralstonia solanacearum* OE1-1 を形質転換し、*in vitro* での誘導期から定常期にかけての増殖能と生物発光に正の相関関係を

示し、OE1-1 と同等の病原性を示す YN5 を得た。青枯病抵抗性品種 LS-89 と感受性トマト品種 大型福寿の接木トマトにおける YN5 由来の生物発光と青枯病の発病について画像解析システム ARGUS50 を装着した VIM カメラを用いて経時的に観察した。根と地際部における *R. solanacearum* 増殖量の程度が青枯病発病に重要な要因であり、LS-89 の青枯病に対する抵抗性は、第一葉下の *R. solanacearum* の増殖抑制に起因することが明らかとなった。LS-89 を子葉下の台木として、大型福寿を穂木として用いた接木トマトでは、*R. solanacearum* はいずれの接木トマトの台木においても潜在感染しており、未発病の接木トマトの穂木からも *R. solanacearum* は検出された。一方、*R. solanacearum* が穂木で著しく増殖した接木トマトはすべて萎凋・枯死した。すなわち、抵抗性台木 LS-89 の *R. solanacearum* に対する何らかの増殖抑制機能が、感受性穂木 大型福寿における *R. solanacearum* の増殖にも影響を及ぼし、青枯病の発病率を量的に調整していることが示唆された。