Evaluation of *Ralstonia solanacearum* Infection Dynamics in Resistant and Susceptible Pepper Lines Using Bioluminescence Imaging

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Abstract

Bacterial wilt, incited by *Ralstonia solanacearum*, is a major disease affecting pepper (*Capsicum annuum*) production worldwide. The most effective management tactic is the deployment of wilt-resistant varieties. However, the lack of a nondestructive method to measure invasiveness and spatio-temporal distribution of *R. solanacearum*, a vascular pathogen, in planta limits better understanding of pepper resistance and plant-pathogen interactions. We evaluated the resistance of 100 pepper lines using *R. solanacearum* strain Rs-SY1 (phylogroup I, isolated from a sweet pepper in South China). Based on the disease severity index (DSI) values, the elite inbred line BVRC 1 and the small-fruited accessions PI 640435 and PI 640444 were identified as resistant (DSI: 1.2, 1.8, and 1.9 out of 4.0, respectively). In order to evaluate bacterial infection dynamics in planta in real time, we generated seven bioluminescent

R. solanacearum strains (BL-Rs1 to BL-Rs7) using vector pXX3 carrying *luxCDABE* genes, and selected BL-Rs7 for inoculation due to its similarity with parent strain Rs-SY1 in morphology, pathogenicity, and highest light emission in vitro. Luminescence intensity was strongly correlated to bacterial population in planta ($R^2 = 0.88$). The utility of the bioluminescence assay was validated by comparing *R. solanacearum* infection dynamics in real-time in vivo between resistant line BVRC 1 and susceptible line BVRC 25. The distribution and multiplication of BL-Rs7 strain in resistant line BVRC 1 was conspicuously limited in plants inoculated in either roots or stem compared with susceptible line BVRC 25. These results suggest that pepper line BVRC 1 may resist colonization by interfering with *R. solanacearum* multiplication in the roots and stem.

Ralstonia solanacearum is a gram-negative soilborne bacterium that causes bacterial wilt in more than 200 plant species in over 50 plant families, including economically important solanaceous crops such as tomato (*Solanum lycopersicum*), potato (*S. tuberosum*), eggplant (*S. melongena*), and pepper (*Capsicum annuum*) (Grimault and Prior 1993, 1994; Lebeau et al. 2011). The bacterium enters host plants through natural openings or wounds in the root system, rapidly colonizes the xylem vessels, and releases large amounts of exopolysaccharide (EPS) that prevent water flow, resulting in wilting symptoms and eventual death of the infected plants (Genin and Denny 2012). *R. solanacearum* strains are partitioned into five races (Buddenhagen et al. 1962; He et al. 1983; Pegg and Moffett 1971), six biovars (Hayward 1964, 1991, 1994; He et al. 1983), and four phylogenotypes (Fegan and Prior 2005). On the basis of phylogenetic analysis, 286 strains of *R. solanacearum* collected from 13 provinces in China were classified as phylogenotypes I and II (Xu et al. 2009).

Bacterial wilt is widely prevalent in peppers across much of Asia (Shekhawat et al. 1992; Yoon et al. 1989). Disease management is difficult because of the broad host range, widespread distribution, and vast genetic variability of the pathogen (Murakoshi and Takahashi 1984). Consequently, selection and planting of resistant varieties of

pepper is considered the most effective approach to manage the disease. Bacterial wilt-resistant accessions identified from global *Capsicum* genetic resources are mainly small-fruited and pungent pepper types (Sarath Babu et al. 2011). However, the resistance found in some accessions could be overcome by a highly virulent KP9547 strain (Mimura et al. 2009). Grimault and Prior (1994) reported *R. solanacearum* causes latent infections in the resistant pepper cultivar Narval, but the plants displayed a high tolerance to bacterial invasion of the stem vascular system, resulting in very weak wilting symptoms. Therefore, the degree of colonization of asymptomatic plants could be used as a criterion for evaluating pepper resistance to bacterial wilt. Several studies showed that presence and density of *R. solanacearum* in different parts of the stem of symptomatic or latently infected hosts was highly variable (Swanson et al. 2005), demonstrating that random stem sampling is an unreliable method of estimating *R. solanacearum* infection. To improve the accuracy of host resistance screening, it is extremely important to develop methods that allow for easy tracking and quantification of bacterial colonization and latency in plants (Cruz et al. 2014).

Bioluminescence genes allow tagging of organisms to monitor and quantify their growth in planta. Strains of *Pseudomonas syringae*, *Erwinia ananas*, *E. amylovora*, *E. tracheiphila*, and *R. solanacearum* transformed with and expressing *lux* genes were used to track bacterial movement and distribution in host plants, and to evaluate host resistance to the corresponding pathogens (Bogs et al. 1998; Dane and Shaw 1996; Fan et al. 2008; Hasegawa et al. 2003; Paynter et al. 2006; Vrisman et al. 2016). With the development of low light imaging techniques, the *luxCDABE* reporter system is particularly advantageous for tracking vascular pathogens. For example, a bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* strain was used to monitor the bacterial colonization process in germinating tomato seeds and seedlings in vivo in real time (Xu et al. 2010, 2012). Recently, Cruz

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary tables and four supplementary figures are available online.

Accepted for publication 24 September 2016.

et al. (2014) genetically modified *R. solanacearum* strain UY031 by insertion of *luxCDABE* operon into its chromosome to compare the behavior of potato accessions with contrasting resistance to bacterial wilt. Classical disease phenotyping combined with the use of luminescence detection could facilitate the evaluation of host resistance to *R. solanacearum*.

Understanding the colonization dynamics of *R. solanacearum* inside its hosts is essential to elucidate the interaction between pathogen and host, and to develop approaches to manage the disease. The objectives of this study were to: (i) evaluate pepper lines for resistance against a phylotype I *R. solanacearum* strain; (ii) develop a bioluminescent strain of *R. solanacearum* marked with the *lux* operon; (iii) compare *R. solanacearum* infection dynamics in the plants of resistant and susceptible pepper lines. The developed bioluminescence imaging method and newly identified bacterial wilt-resistant line should be useful for pepper breeding as well as for identification and characterization of potential resistance genes.

Materials and Methods

Isolation and maintenance of *R. solanacearum*. Twenty symptomatic sweet pepper plants infected by *R. solanacearum* were collected from a commercial field in Sanya (109°50'E, 18°20'N), Hainan, China, in November 2014. All symptomatic basal stems were washed thoroughly in running water, surface sterilized with 70% ethanol for 30 s, and rinsed two times in sterile distilled water. Transversely cut stem samples (2-cm length) were placed in 5.5-cm diameter petri dishes after addition of 5 ml of sterile water for 3 h to allow the bacterial cells to ooze from the cut surface. Exudates from the stem were serially diluted with sterile distilled water, streaked on Kelman's triphenyl tetrazolium chloride (TZC) medium, and incubated at 28 to 30°C for 48 h (Kelman 1954; Xu et al. 2009). Putative *R. solanacearum* colonies that were mucoid and egg-shaped with pink-reddish center were selected and purified by restreaking on TZC medium for 3 days at 28°C. A single colony, designated Rs-SY1, was selected and maintained in cryogenic vials (Corning Incorporated, Mexico) containing 1 ml sterile distilled water at room temperature.

Molecular identification of *R. solanacearum*. Total genomic DNA of Rs-SY1 strain was extracted following the protocol of TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa, Dalian, Liaoning, China), and then used as the template for the endoglucanase (*egl*) gene and 16S rDNA amplification using a GoTaq Green Master Mix Kit (Promega, Madison, WI) as previously described (Horita and Tsuchiya 2000; Ivey et al. 2007; Poussier et al. 2000). The resulting PCR products were separated on 1.5% agarose gel and purified using an Agarose Gel Purification Kit (Biomed Co., Beijing, China). At least five purified PCR products from each of the *egl* gene and 16S rDNA were sequenced at Sunbiotech Company (Beijing, China). Partial nucleotide sequences of 16S rDNA and the *egl* gene from Rs-SY1, with accession numbers KT748524 and KT762388, respectively, were deposited in the GenBank database.

Phylotype affiliation of Rs-SY1 was determined using phylotype-specific-multiplex PCR (Pmx-PCR) (Fegan and Prior 2005; Wicker et al. 2007). Each 25- μ l reaction mixture contained 50 ng of template DNA; 0.5 μ l primers of 759 and 760 (10 μ M); 0.75 μ l primers of Nm12:21:1F, Nm12:21:2F, and Nm12:22:InF (10 μ M); 1.75 μ l primer of Nm12:23:AF (10 μ M); 1 μ l primer of Nm12:22:RR (10 μ M); and 12.5 μ l GoTaq Green Master Mix Kit (Promega, Madison, WI). Reactions were heated at 96°C for 5 min followed by 36 cycles of 94°C for 15 s, 59°C for 30 s, 72°C for 30 s; and then a 10-min final extension at 72°C. A phylogenetic neighbor-joining tree based on the partial coding (687-bp) *egl* gene sequence was conducted with Rs-SY1 and 26 reference strains selected from the literature (Prior and Fegan 2005; Wicker et al. 2007). The NCBI accession numbers of those reference strains are summarized in Supplementary Table S1.

Construction of bioluminescent *R. solanacearum* strains. Transformation was achieved by mixing 100 μ l of electrocompetent *R. solanacearum* strain Rs-SY1 with 1 μ g of the suicide plasmid pXX3 carrying the *Cmx::luxCDABE::Tn1409* cassette (Xu et al. 2010). Competent cells for Rs-SY1 strain and electroporation-transformation were prepared as previously described (Xu et al. 2010) with the

following electroporation parameters: 1.8 KV voltage; 25 μ F capacitance; 600 Ω resistance. Immediately after electroporation, the cells were mixed with 0.6 ml nutrient yeast extract broth (NBY) and incubated with shaking (140 rpm) for 3 h at 28°C. After incubation, 100 μ l aliquots of the suspension were spread on TZC medium supplemented with chloramphenicol (45 μ g/ml), incubated for 2 days at 28°C, and the bioluminescent transformants were examined under a CCD camera in an in vivo imaging system (Berthold NightSHADE, Model LB985) (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

Assessing bioluminescence intensity in vitro. Single colonies of each of seven BL-Rs strains and the Rs-SY1 strain growing on NBY agar were individually inoculated into 200 ml NBY medium and incubated at 28°C with shaking at 120 rpm on a rotary shaker (Minitron, Switzerland). Measurements of optical density at 600 nm and light intensity were taken at 6, 12, 24, 36, 48, and 72 h. A Thermo Biomate 3S UV-visible spectrophotometer was used to measure the OD₆₀₀ from a 1 ml subsample of each culture. To quantify the amount of light emitted by the transformed BL-Rs strains, three replicates of 200 μ l of culture were transferred to a white 96-well plate (Thermo Scientific, Waltham, MA), and the relative light units (RLU) from each well were determined using TriStar² (Berthold, Model LB942) with ICE software (version 1.04). The RLU for each well was normalized for background luminescence by subtracting the value of a control well containing nutrient broth only. Bioluminescence intensity was compared among BL-Rs strains at an optical density of 0.12. The experiment was conducted twice with three biological replications. The RLU data were log-transformed and subjected to analysis of variance (ANOVA) using SPSS Statistics 20.

Pathogenicity test of bioluminescent strain. *R. solanacearum* strain Rs-SY1 and bioluminescent strain BL-Rs7 were cultured on nutrient agar plates at 28°C for 48 h. Bacterial suspensions were prepared in sterile distilled water and adjusted to OD₆₀₀ = 0.18 (approximately 2×10^8 CFU/ml).

Seeds of pepper line BVRC 25 were sown into 72-Cells Plug Tray (54 cm long $\times$ 28 cm wide $\times$ 5.5 cm deep) (Taizhou Longji Yuanyi Cailiao Co., Ltd., Zhejiang, China) containing a mixture of peat and vermiculite (2:1) after sterilization in an autoclave (ALP Co., Ltd., Tokyo, Japan) for 30 min. Plants were grown in a greenhouse at 28/25°C day/night cycle under a 14-h photoperiod, and fertilized with Poly-feed (1 g/liter) (Haifa Chemicals Ltd., Haifa Bay, Israel) weekly. Seedlings in the four-expanded-leaf stage were carefully removed from 72-cell flats, and roots were rinsed in running water. Twenty plants were inoculated by directly dipping the roots in the Rs-SY1 or BL-Rs7 suspension for 30 min, then were transplanted into individual 10 cm (diameter) $\times$ 10 cm (height) pots with sterilized peat and vermiculite. Each plant was considered a replicate. Pots were arranged in a completely randomized design in a growth chamber (27/25°C day/night cycle with a 14-h photoperiod). Water and fertilizer were provided as needed.

Disease severity of each plant was scored 8 days after root-dip inoculation according to the following criteria: 0 = asymptomatic; 1 = minor symptoms with less than 20% wilted leaves; 2 = moderate symptoms with 20 to 50% wilted leaves; 3 = severe symptoms with 50 to 80% wilted leaves; 4 = dead plant. For comparison of pathogenicity between Rs-SY1 and BL-Rs7 strains, the inoculated plants were rated at 1, 3, 5, 7, 9, 11, 13, and 15 days after root-dip inoculation. The disease severity index (DSI) for pepper line BVRC 25 inoculated with Rs-SY1 or BL-Rs7 was calculated as follows: $DSI = \sum(\text{disease score} \times \text{number of plants with that disease score}) / \text{total number of plants}$. The experiment was repeated three times. Student's *t*-test was used to identify whether there were significant differences in pathogenicity between Rs-SY1 and BL-Rs7 strains.

Bacterial wilt screening and resistance evaluation. One hundred pepper lines, including 25 elite inbred lines from Beijing Vegetable Research Center (BVRC) in Beijing, China, and 75 lines provided by the U.S. National Plant Germplasm System (NPGS) (Supplementary Table S2), were evaluated for resistance to *R. solanacearum* strain Rs-SY1. The method for inoculum preparation,

plant growth, and root inoculation was the same as for the pathogenicity test described above. Disease severity of each plant was scored at 3, 6, 9, 12, 15, 20, 25, 30, 35, and 40 days after root-dip inoculation. The experiment was conducted twice, and the experimental design was a randomized complete block with 10 plants for each pepper line. Each plant was considered a replicate.

The area under the disease progress curve (AUDPC) was calculated using the *agricolae* R package (Mendiburu 2014) based on the assessed plant disease score. The disease severity index (DSI) for each line was calculated at the end of the experiments. The disease response was based on the plant score at the end of the two experiments. If the DSI in both experiments was <2, the line was considered resistant (R); if the DSI in either of the experiments was >2, the line was considered susceptible (S).

Bioluminescence imaging of roots and stems of inoculated plants. Bioluminescent *R. solanacearum* strain BL-Rs7 was selected due to its similarity with parent strain Rs-SY1 in morphology and pathogenicity, and highest light emission in vitro, and used to compare bacterial invasiveness and spatio-temporal distribution in pepper lines BVRC 1 and BCRC 25 with root dipping and stem inoculation methods. Ten plants of each line at the four-expanded-leaf stage were inoculated with BL-Rs7 strain (2×10^8 CFU/ml) using the root dipping method described above. Stem inoculation was performed on each line at the six-expanded-leaf stage. Five seedlings for each line were inoculated just above the cotyledon by cutting the stem to one-third of its diameter with a blade, and a drop of 10- μ l bacterial suspension (1×10^5 CFU/ml) was placed on the wound using a 2-ml syringe.

To facilitate real-time imaging, the inoculated plants were transplanted into pots (8 cm $\times$ 8 cm, diameter $\times$ height) filled with sterilized perlite, and watered with 0.1 $\times$ Hoagland's nutrient solution (Hoagland and Arnon 1950). Pots were arranged in a completely randomized design in a growth chamber (27/25°C night/day cycle with a 14-h photoperiod). Plant colonization by BL-Rs7 was imaged using a Berthold NightSHADE CCD camera (Model LB985). Bioluminescent images were obtained using the procedures described by Xu et al. (2012). Due to the high sensitivity of the CCD camera, plant phosphorescence was detected immediately after the plants were placed in the imaging chamber. Therefore, pepper seedlings were placed in the dark lightproof chamber at room temperature for over 30 min to quench plant phosphorescence. After dark incubation, pepper seedlings were carefully removed from pots, gently shaken to remove the attached perlite, and placed in the lightproof imaging chamber. The imaging parameters of the sensitivity/resolution setup were 4 $\times$ 4 binning, and 5 min delay in darkness imaging chamber prior to an incremental exposure time of 180 s. The imaged plants were replanted into the pots and placed in the growth chamber. The root-inoculated seedlings were imaged to monitor the movement of bacteria daily over a 10-day time-course of BL-Rs7-inoculation. Stem-inoculated plant colonization by BL-Rs7 was imaged at 2, 4, 5, 6, 7, 9, and 18 days post inoculation (dpi). The bioluminescence signal in planta was quantified with indiGo software (Berthold Technologies, Oak Ridge, TN), and the light intensity was displayed in counts per second (cps). The experiments were conducted three times.

Determining the bacterial population in plants. Two experiments, each with three biological replicates, were conducted to determine the bacterial populations in plants of pepper lines BVRC 1 and BVRC 25 at the six-expanded-leaf stage. The methods for plant growth and root dipping inoculation with BL-Rs7 strain were described above. At 1, 3, 5, 7, 9, 11, and 13 dpi, five plants each of pepper line were rinsed with sterile distilled water, and sampled by cutting 2-cm length root, and 0.5-cm length collar (just above the soil surface), midstem, and apical-stem. Populations of *R. solanacearum* in samples were determined using procedures described by Ishihara et al. (2012) and Dai et al. (2016). Samples were weighed separately and homogenized using glass sticks in test tubes with 1 ml of sterile distilled water. Each homogenate was serially diluted with sterile distilled water and plated on TZC medium amended with chloramphenicol (45 μ g/ml). The plates were incubated at 28°C for 2 days. Colonies recovered from plant tissues in the two pepper lines were counted, and bacterial population (CFU/g of fresh weight) was

log-transformed. In no case did we detect any significant variation in bacterial populations obtained from the two experiments of individual stages. Bacterial populations (log CFU/g) recovered from roots, collar, midstem, and apical-stem were compared between pepper lines BVRC 1 and BVRC 25 by one-way ANOVA ($P < 0.05$) using SPSS Statistics 20.

Results

Morphological and phylotype identification of *R. solanacearum* strain Rs-SY1. On TZC, the colony morphology of *R. solanacearum* strain Rs-SY1 was mucoid, egg-shaped, and white with a pink center, sharing the characteristics of virulent strains of *R. solanacearum* (Zheng et al. 2014). The expected 280-bp *R. solanacearum* species-complex-specific amplicon and a 150-bp phylotype-I-specific amplicon were generated from Rs-SY1 strain by Pmx-PCR (data not shown). Phylogenetic analysis of *egl* gene sequences indicated that the Rs-SY1 strain belongs to the major group found within the phylotype I strains (Supplementary Fig. S1). BLAST sequence comparisons indicated that the portion of the *egl* sequence (698 bp) of Rs-SY1 was 100% identical to reference strain CFBP765 (GenBank: EF371810) isolated from tobacco in Japan (Wicker et al. 2007).

Generation of bioluminescent *R. solanacearum* strains. Bioluminescent colonies were obtained from each of seven electroporation-transformations conducted with strain Rs-SY1 and plasmid pXX3. The transformation efficiency was on average about 80 transformants/ μ g of vector pXX3 on nutrient agar containing 45 μ g/ml chloramphenicol, and an average of 12 colonies emitted luminescence signals under the CCD camera. One bright bioluminescent colony from each transformation assay was selected, and these bioluminescent strains were serially coded as BL-Rs1 to BL-Rs7 (Supplementary Fig. S2). Notably, among the seven mutant strains, BL-Rs1 strain exhibited a distinct morphology compared with other transformed strains, with a red colony and small white edge on TZC medium; colonies of the other mutant strains had a red center with white edge identical to wild type Rs-SY1 strain. Upon further analysis using PCR, the presence of *Cmx* and *luxC* gene were confirmed in all luminescent strains (data not shown), indicating that the *lux* genes were successfully transferred into the Rs-SY1 strain.

Identification of pathogenic and highly luminescent *R. solanacearum* strains. Intensity of bioluminescence from whole *lux*-tagged strains positively correlated with bacterial growth from the lag phase to the stationary phase in vitro (Supplementary Fig. S3). To select highly bioluminescent strains, luminescence intensity of the *lux*-tagged strains BL-Rs2 to BL-Rs7 was compared at mid-exponential growth phase at approximately 2×10^8 CFU/ml. BL-Rs7 strain exhibited the highest luminescence among all the strains ($P < 0.01$) (Fig. 1). Plants of pepper line BVRC 25 inoculated with BL-Rs7 exhibited disease progression similar to those inoculated with wild type Rs-SY1 (Supplementary Fig. S4). Thus, there was no significant difference between wild-type virulent Rs-SY1 and *luxCDABE*-tagged BL-Rs7 in studies of *R. solanacearum*-pepper interactions.

Regression analysis showed a significant positive linear relationship between bacterial population and bioluminescent intensity in stems ($R^2 = 0.88$) (Fig. 2), indicating that stronger signals corresponded to higher bacterial populations in planta. Light emission of BL-Rs7 strain could be clearly detected with 8×10^7 bacterial cells per gram fresh weight of pepper stem tissue.

Evaluation of pepper lines for resistance to bacterial wilt. One hundred pepper lines comprising five cultivated species: *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens*, were evaluated for resistance to *R. solanacearum* strain Rs-SY1. Some pepper lines were known to be resistant to plant pathogens, including bacterial spot-resistant lines PI 640528 and PI 640529, Tomato spotted wilt virus (TSWV)-resistant lines PI 152225 and PI 159236, bacterial wilt-partial resistant lines PI 645539 (Ishii-Midori), and *Phytophthora capsici*-resistant line PI 201234. Bacterial wilt-susceptible line PI 586661 and a universally susceptible line ISF 1 01 (Early Cal Wonder) (Mimura et al. 2009; Sarath Babu et al. 2011) were also included.

The correlation of AUDPC between two experiments for these lines was highly significant with the correlation coefficient $R^2 = 0.76$

(data not shown). The average AUDPC displayed continuous variation among the accessions (Fig. 3). Complete resistance (no wilting symptoms) was not observed but the pepper line BVRC 1 had the fewest disease symptoms and lowest AUDPC (average AUDPC = 17.0) compared with other accessions, whereas the accession exhibiting the greatest susceptibility was ISF 1 01 (average AUDPC = 147.7). The symptoms progressed to 100% mortality rate in ISF 1 01 within 4 dpi, suggesting that Rs-SY1 is an aggressive pathogen of pepper. The proportion of dead plants was significantly less in BVRC 1 compared with other lines. Under high disease pressure in two experiments, the mean DSI values for *C. annuum* lines BVRC 1, PI 640435, and PI 640444 were 1.2, 1.8, and 1.9 out of 4.0, respectively. Therefore, these three pepper lines were considered resistant to *R. solanacearum* strain Rs-SY1 on the basis of mean disease index score lower than 2.0.

Visualization of bioluminescent *R. solanacearum* colonization of resistant and susceptible pepper lines. In plants inoculated by root dipping, a few bioluminescent signals were detected in the roots of both BVRC 1 and BVRC 25 plants 2 dpi (Fig. 4). Strong luminescence signals were detected in the collar and stem of BVRC 25 plants from 3 dpi and increased gradually over time, while no signals were detected in collar or stem of BVRC 1 plants. Systemic colonization of apical-stems was obvious 5 dpi in BVRC 25 plants and minor wilting symptoms were noticed. As the wilting progressed, the intensity of luminescence increased significantly in BVRC 25 plants until 7 dpi and subsequently remained at a high level through 10 dpi (data not shown). Neither wilting nor luminescence signals were detected in BVRC 1 plants from 4 to 10 dpi.

Bacterial titer was low in the roots of susceptible and resistant plants 1 dpi, and under the detection limit of bioluminescent imaging (Table 1; Fig. 4). Bacterial titers were approximately 10^1 , 10^2 , and 10^7 times higher in the roots, collar, and midstem, respectively, of BVRC 25 plants compared with corresponding tissues of BVRC 1 plants 3 dpi. Bacterial populations in resistant pepper line BVRC 1 plants increased slowly in the roots and collar and reached a peak at 9 dpi, then decreased by 11 and 13 dpi; whereas in the susceptible pepper line BVRC 25, bacteria multiplied extensively in all plant tissues and quickly increased to 10^8 CFU/g 5 dpi and reached 10^{10} CFU/g before the death of plant (Table 1).

Bioluminescent signals were detected in stem-inoculated susceptible BVRC 25 plants from 5 dpi, and bacteria were observed both

above and below the point of inoculation (Fig. 5). However, *R. solanacearum* multiplication in stems was conspicuously retarded in resistant BVRC 1 plants until 9 dpi. At 18 dpi, BVRC25 plants completely wilted, while all BVRC 1 plants remained asymptomatic; three out of 10 plants showed detectable bioluminescent signals in the stem.

Discussion

Breeding programs aimed at developing wilt-resistant pepper varieties are hampered by the scarcity of sources of resistance to *R. solanacearum*. In this study, 100 pepper accessions were evaluated in

Fig. 2. Regression analysis of bioluminescent intensity versus bacterial population recovered from pepper stems after inoculation with *Ralstonia solanacearum* BL-Rs7 strain. Data points were bacterial populations and bioluminescence intensities from 0.5 cm above the cotyledons in BVRC 25 plants. The bioluminescence intensity data were indicated by counts (cts).

Fig. 1. Comparison of luminescence intensity among *lux*-tagged *Ralstonia solanacearum* strains. Broth cultures of *R. solanacearum* strains were grown in NBY medium, and luminescent intensity was measured at an optical density OD₆₀₀ = 0.12. The error bars indicate standard deviations, and values on the error bars with same letter were not significantly different by one-way analysis of variance (ANOVA, $P < 0.01$).

controlled conditions using root inoculation of pathogenic strain Rs-SY1 isolated from Sanya, China. Two experiments resulted in repeatable results, with a significant correlation, attesting to the reliability of the root-dip inoculation procedure and the stability of resistance in the hot pepper lines BVRC 1, PI 640435, and PI 640444. Many bacterial wilt-resistant pepper lines have been identified but have not been extensively exploited, primarily because of their small fruit size.

On the contrary, the elite inbred line BVRC 1 exhibited acceptable horticultural characters (average fruit length 31 cm, average fruit diameter 1.5 cm, and average fruit weight 25 g), and may be applied effectively in breeding for linear-fruited and pungent varieties of pepper that are popular in open field cultivation in the south of China (Zhao et al. 2013). However, BVRC 1 needs to be further screened against isolates that represent a greater geographic distribution, due

Fig. 3. Comparison of resistance against *Ralstonia solanacearum* strain Rs-SY1 in 100 pepper lines. The average AUDPC for each line was calculated from two experiments. Pepper lines BVRC 1 and BVRC 25, shown by circles, were used for subsequent real-time visualization of bioluminescent strain behavior in planta.

Fig. 4. Real-time visualization of *Ralstonia solanacearum* strain BL-Rs7 colonization of resistant pepper line BVRC 1 and susceptible line BVRC 25. Roots of 10 plants of each pepper line were inoculated with BL-Rs7 at a concentration of 2×10^8 CFU/ml. Representative bioluminescent images of the infected pepper lines BVRC 25 (left) and BVRC 1 (right) photographed using Berthold NightSHADE CCD camera. The color-coded bar displays the intensity scale of the measurement.

to recognized virulence variation among isolates (Gutarra et al. 2015; Mimura et al. 2009; Wicker et al. 2007).

An earlier study showed that *R. solanacearum* resistance evaluation should not only focus on wilting symptoms but also on latent infection (Lebeau et al. 2011). A luminescent variant of *R. solanacearum* UY031, a phylotype IIB strain highly aggressive on potato (Siri et al. 2011), was applied to detect latency in symptomless potato (Cruz et al. 2014). In this study, to monitor the movement of phylotype I strain Rs-SY1 in pepper, the bacterium was marked with the *lux* operon from the modified *Tn1409*-containing vector pXX3. Previously, pXX3 had a very low transformation frequency in the gram positive *C. michiganensis* subsp. *michiganensis* chromosome (Xu et al. 2010). In this study, *R. solanacearum* Rs-SY1 strain electroporation with pXX3, following similar electroporation parameters used in *C. michiganensis* subsp. *michiganensis*, resulted in a high number of luminescent transformants in seven individual experiments. Vrisman et al. (2016) also used pXX3 to develop a *Cucumis*-derived bioluminescent strain of *Erwinia tracheiphila* marked with the *lux* operon. These results suggest that pXX3 may be useful in transforming other gram negative bacteria with the *lux*-operon.

Since integration of *Tn1409* in the bacterial genome is nonspecific, the *lux* operon may be integrated down-stream of promoters that are expressed at different levels, which explains why the seven bioluminescent strains BL-Rs1 to BL-Rs7 emitted varying light intensities in vitro (Fig. 1). The strongly bioluminescent strain BL-Rs7 also showed similar growth and pathogenicity as the parental wild-type strain Rs-SY1; moreover, a minimum 8×10^7 bacteria per gram of fresh weight could be detected by bioluminescent signals in planta (Fig. 2). The minimum detection limit of BL-Rs7 in vitro was 1×10^5

CFU. In latently infected resistant tomato plants, the multiplication of *R. solanacearum* in the upper hypocotyl reached 9.2×10^5 CFU/g in Hawaii 7996 and 9.3×10^6 to 1.6×10^8 CFU/g in 10 other resistant cultivars 14 dpi (Nakaho et al. 2004). *R. solanacearum* densities in the roots approached saturation at 9 dpi with approximately 1×10^8 CFU/g in mulberry resistant cultivar KQ10 and YS283 (Dai et al. 2016). Since *R. solanacearum* populations can reach very high levels in resistant hosts, this level of sensitivity is generally adequate to evaluate latent infections in pepper germplasm. The detection limit can be improved by extending the exposure time of the CCD camera as well as by adjusting photo properties of indiGo software. Theoretically, a single luminescent bacterial cell is possibly detectable using device-enhanced microscopy (Silcock et al. 1992).

Numerous studies have used external symptom recording to screen and evaluate pepper resistant genotypes, but whether the non-wilting plant was latently infected was not determined. Latent infection is widespread and has been identified in several asymptomatic host plants including tomato, geranium, squash, and potato (Cruz et al. 2014; Hikichi et al. 1999; Swanson et al. 2005; Vrisman et al. 2016). We have successfully visualized the *lux*-labeled *R. solanacearum* multiplication and translocation in resistant and susceptible pepper plants before any symptoms appeared (Figs. 4 and 5). Thus, under our experimental conditions, bioluminescent imaging could be used to identify latent infections in symptomless pepper plants.

Bioluminescent images and populations of *R. solanacearum* recovered from the root-inoculated plants suggest that bacterial invasion through roots was not inhibited in resistant line BVRC 1, but bacterial proliferation was retarded and restricted in the roots and collar of these plants compared with those of the susceptible line BVRC

Table 1. Comparison of bacterial populations recovered from different tissues of plants of resistant pepper line BVRC 1 and susceptible line BVRC 25 after root inoculation with bioluminescent *Ralstonia solanacearum* strain BL-Rs7

Days post inoculation	Roots		Collar		Midstem		Apical-stem	
	BVRC 1	BVRC 25	BVRC 1	BVRC 25	BVRC 1	BVRC 25	BVRC 1	BVRC 25
1	6.14 a ^y	6.69 a	4.19 a	4.23 a	0 a	0 a	0 a	0 a
3	6.53 a	7.65 b	4.95 a	7.51 b	0 a	7.34 b	0 a	0 a
5	7.36 a	8.82 a	5.50 a	8.78 b	0 a	8.64 b	0 a	6.06 b
7	7.24 a	9.36 b	5.34 a	9.64 b	0 a	8.46 b	0 a	8.06 b
9	7.12 a	9.59 b	5.70 a	10.42 b	0 a	10.07 b	0 a	9.51 b
11	6.76 a	9.59 b	4.24 a	10.18 b	0 a	10.31 b	0 a	10.57 b
13	5.99	ND ^z	4.69	ND	0 a	ND	0 a	ND

^y Bacterial populations (log CFU/g) recovered from roots, collar, midstem, and apical-stem were compared between pepper lines BVRC 1 and BVRC 25 at 1, 3, 5, 7, 9, 11, and 13 dpi. Means ($n = 10$) followed by same letter in a row under same plant tissue were not significantly different by one-way analysis of variance (ANOVA $P < 0.05$).

^z Not determined as the plant completely wilted.

Fig. 5. Real-time visualization of *Ralstonia solanacearum* strain BL-Rs7 proliferation in the stems of susceptible pepper line BVRC 25 (top row) and resistant line BVRC 1 (bottom row). Stems of five plants of each of these pepper lines were inoculated with bioluminescent strain BL-Rs7. Bioluminescent imaging of the infected plants was conducted using a Berthold NightSHADE CCD camera. The color-coded bar displays the intensity scale of the measurement.

25. No bioluminescence was observed nor were any bacteria isolated from the stem of resistant line BVRC 1 within 10 dpi of roots. However, luminescent signals were detected in the stem of 30% of stem-inoculated BVRC 1 plants at a late infection stage (18 dpi). Thus, bacterial multiplication in the stem was limited but not completely suppressed in BVRC 1 plants. Similar results were reported on *R. solanacearum* colonization patterns in tomato resistant cultivar Hawaii 7996 (Grimault et al. 1995). A much lower colonization rate and bacterial density was found in the midstem than those in the collar. Therefore, restriction of bacterial multiplication in roots plays the primary role in BVRC 1 resistance against *R. solanacearum*. In conclusion, our study verified that pepper resistance to bacterial wilt was not correlated with inhibition of root invasion but largely was associated with limitation of bacterial multiplication in the roots and stem.

Acknowledgments

The work was funded by the Research Projects of Beijing Academy of Agriculture and Forest Sciences (QNJJ 201510 & KJCX20150202), Beijing Municipal Science & Technology Commission (Z141100002314019), and Beijing Innovation Team Building Project (GCTDZJ2014033002). The authors thank the United States Department of Agriculture (USDA) for providing some of the pepper lines.

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