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A Real-Time PCR Assay for the Quantitative Detection of *Ralstonia solanacearum* in Horticultural Soil and Plant Tissues

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A specific and rapid real-time PCR assay for detecting *Ralstonia solanacearum* in horticultural soil and plant tissues was developed in this study. The specific primers RSF/RSR were designed based on the upstream region of the UDP-3-*O*-acyl-GlcNAc deacetylase gene from *R. solanacearum*, and a PCR product of 159 bp was amplified specifically from 28 strains of *R. solanacearum*, which represent all genetically diverse AluI types and all 6 biovars, but not from any other nontarget species. The detection limit of 10^2 CFU/g tomato stem and horticultural soil was achieved in this real-time PCR assay. The high sensitivity and specificity observed with field samples as well as with artificially infected samples suggested that this method might be a useful tool for detection and quantification of *R. solanacearum* in precise forecast and diagnosis.

Keywords: Detection, quantification, *Ralstonia solanacearum*, real-time PCR

Ralstonia solanacearum is the pathogen of bacterial wilt on a large range of hosts, which are more than 200 species representing over 50 families of plants including solanaceous crops [6]. The first line of prevention of *R. solanacearum* is the travel restriction of infected plants or seeds. Infected plants could be primarily visually identified, whereas latent infections in plant tissues and propagating stocks and infected seed might escape from this simple inspection. Thus, sensitive detection systems are necessary for controlling the spread of this bacterium [2].

In the past years, the most widely used method to detect *R. solanacearum* in natural substrates is plating a suspension of them on a semi-selective medium and then identifying the colonies grown on it [2, 5]. This traditional method is time-consuming. Meanwhile, the culture method may result in an underestimate of the actual population, for the bacteria including *R. solanacearum* could enter in a viable but nonculturable (VBNC) state [4], which represents a transient inability to grow on nutrient medium, while still being metabolically active [15, 31]. Therefore, many PCR-based methods have been developed for the detection of *R. solanacearum* without culture in recent years. These approaches are usually according to the amplification of ribosomal gene sequences (*i.e.*, 16S or 16S–23S intergenic spacer region of the ribosomal) [17, 18, 28, 30]; however, it may result in some false-positive pattern with related species such as *Ralstonia picketti* because of the high degree of conservation of the ribosomal genes within the genus *Ralstonia* [25]. Some other special primers that targeted different genes such as the endoglucanase gene, *hrpB*, *hrcu*, *flic*, and the DNA sequence of cytochrome *c*1 signal peptide were developed for the detection of *R. solanacearum* with the sensitivity$\geq 10^3$ CFU/g soil [11, 16, 19, 23]. The primers RSAF1 and RSAR1 and probe RSP1 were developed from a unique 329-bp DNA fragment from *R. solanacearum* biovar 4 from ginger and shown to target to all strains of *R. solanacearum* race 1 biovars 3 and 4 but not biovars 1 and 2 [26] . Another fluorogenic (TaqMan) PCR assay was employed in a multiplex reaction for a broad-range detection of all biovars of *R. solanacearum* where the sensitivity was only 10^4 to 10^7 cells/ml for 1:10-diluted potato extract; some compounds of plants probably inhibited this sensitivity [30]. The sensitivity might be promoted by enriching the target bacterium, by culturing samples on a semi-selective medium prior to PCR detection; but it took up to 60 h to achieve a sensitivity of about 10^2 CFU/g infected soil [3, 20, 21]. Phage amplification

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integrated with real-time PCR assay was introduced for *R. solanacearum* detection and reached a limit down to approximately 10^2 CFU/g of soil and plant tissues, but the samples had to be incubated for 1 h to allow the opportunity for phage proliferation and assessed immediately; the phage was not specific to all tested strains of *R. solanacearum* [12].

To date, specific and sensitive primers for the detection of the different strains of *R. solanacearum* on the level of species directly from natural substrates are still not available. The main goal of the present study was to develop specific primers for detecting various strains of *R. solanacearum* specifally and sensitively. For this purpose, we designed primers according to the upstream region of *R. solanacearum* UDP-3-*O*-acyl-GlcNAc deacetylase gene conserved only in this bacterium [16], and then established a real-time PCR (SYBR Green I) method to detect the bacterium in plants and soil samples.

Materials and Methods

Bacterial Strains and Culture

The strains of *R. solanacearum* and other bacteria used to determine the specificity of the primers are shown in Table 1. Most of the control strains were plant pathogenic or isolated from soil samples. All of them are kept by the Department of Plant Pathology, Nanjing Agricultural University, China. The 28 strains of *R. solanacearum* used in the present study were distributed among all of the 13 AluI types and the 6 biovars of this bacterium, which were isolated from 15 species of hosts and different geographical origins including 10 provinces of China and seven other countries [8]. *R. solanacearum* was incubated in YGPA broth at 28°C, and the control bacteria were routinely grown with shaking in Luria–Bertani (LB) broth at 30°C. The total DNA was extracted from the microorganisms using a genomic DNA extraction kit (SBS Co., Shanghai, China).

Collection of Soil and Plant Samples

The two samples of horticultural soil were collected from the cultivated layer of the following fields. The first sample was infested soil of the soybean field in Huai'an City, Jiangsu Province. In this field, tomatoes had been grown for 5 years and the wilt disease caused by *R. solanacearum* had occurred in the three continuous years from 2004 to 2006. Thus, the soybean (non-host plants of *R. solanacearum*) was grown in 2007 and 2008. The second sample was 1 g of the uninfested soil with artificial addition of *R. solanacearum* suspension (1 ml containing 9.5×10^3 CFU). The soil was collected from a tomato field in Nanjing, Jiangsu Province. Another four samples were different tissues of different plants: the stem of tomato without wilt symptom obtained from the infested field in Huai'an; the stems of infested tobacco acquired in our greenhouse in which the tobacco was inoculated with *R. solanacearum* and showed the wilt symptoms; the leaves of healthy mulberry (1 g) collected from Hangzhou, Zhejiang Province, and ground together with *R. solanacearum* suspension (1 ml containing 5.0×10^8 CFU); and healthy tuber bought from a market and inoculated artificially with *R. solanacearum*. To detect the pathogen in ginger tuber, 1 ml of bacterial suspension (*R. solanacearum* containing 5.0×10^8 CFU/ml was inoculated into 1 g of ginger tuber. After incubating the tuber for 1 day at room temperature, it was ground and prepared as described above.

Designing of Primers and Real-Time PCR Amplification

The oligonucleotide primers used for the real-time amplification of *R. solanacearum* were designed based on the sequence of the upstream region of the *R. solanacearum* UDP-3-*O*-acyl-GlcNAc deacetylase gene. The potential target regions were selected by identifying the sequence highly conserved among all the tested strains using the Clustal W program available in the Lasergene 5 package (DNAStar, U.S.A.). The primers RSF (5' GTGCCTGCCTCCAAAACGACT 3') and RSR (5' GACGCCACCCGCATCCCTC 3') were designed with a predicted PCR product of 159 bp by Primer Premier 5.0. The real-time PCR amplifications were performed with the DNA Engine Opticons 4 System (MJ Research,Waltham, MA, U.S.A.) using the SYBR Green I fluorescent dye detection in 20-μl volumes containing 10 μl of SYBR Premix Ex *Taq* mix (TaKaRa Biotech.Co., Japan), 2 μl of template, and 0.4 μl of both forward and reverse primers (10 mM each). Two reaction steps and the following parameters were used: an initial preheat for 2 min at 95°C, followed by 40 cycles at 95°C for 20 s, 62°C for 25 s, 72°C for 35 s, and 85°C for 3 s. The treatment of 85°C was employed in order to detect and quantify the fluorescence at a temperature above the denaturation of primer-dimers. Once the amplifications were completed, the melting curves were obtained based on a standard protocol (refer to manual) and used to identify the characteristic peak of PCR product. Data were analyzed by using the MJ Opticon Monitor 3 software. Each sample was replicated twice, and this experiment was repeated twice.

Specificity of Real-Time PCR

To confirm the specificity of the primers to *R. solanacearum*, the template DNAs extracted from all of the 28 strains of *R. solanacearum* and 15 control bacterial strains (Table 1) were used in real-time PCR assay. The same volume of sterile distilled water without the template DNA was used as the negative control in each PCR run. All reactions were performed in triplication. The PCR-amplified products were visualized on 2% agarose gel and stained with ethidium bromide.

Detection of *R. solanacearum* from the Soil and Plant Samples

To quantify the *R. solanacearum* from samples, standard curves of real-time PCR for the detection of *R. solanacearum* from the plants and soil samples were generated. The culture broth of strain GMI1000 was adjusted to 1×10^7 CFU/ml suspension with sterile distilled water, and a 10-fold dilution series was made from each suspension (10^7–10^0). The number of the cells in the serial dilution was confirmed by incubation on TZC agar plates at 30°C for 3 days.

The *R. solanacearum* DNA was extracted from tomato according to the following steps: (1) 100 μl of bacterial suspension from each dilution and 0.1 g of healthy tomato stem collected from the greenhouse were transferred into a 1.5-ml centrifuge tube, to which 400 μl of 0.5 M NaOH was added; (2) the samples were crushed acutely by a FastPrep FP101 beater (Bio 101), placed in a rack for 5 min to split the cells, and then centrifugated at 12,000 rpm for 3 min; (3) the supernatant with DNA was removed into a fresh centrifuge tube (1.5 ml) and cro-tube A of soil, and further purified using the UNIQ gel extraction kit (Sangon Co., Shanghai, China). Two μl of DNA extraction from each sample was subjected to PCR

Table 1. Strains of *Ralstonia solanacearum* and other species used in this study.

Strains of *Ralstonia solanacearum*					
No.	Isolates	Biovar	Host	Geographical origin	Source[a]
1	UW278	1	*Nicotiana tabacum*	Mexico	D
2	K60	1	*Lycopersicon esculentum*	Wake Co., N.C., U.S.A.	D
3	UW40	1	*Musa* sp.	Honduras	D
4	UW134	1	*Solanum tuberosum*	Kenya	D
5	GD1993C1	2	*Casuarina equisetifolia*	Guangdong, China	C
6	FJE1	2	*Solanum melongena*	Fujian, China	C
7	UW265	2	*Nicotiana tabacum*	Taiwan, China	D
8	I35	2	*Pelargonium capitatum*	U.S.A.	D
9	HB512	3	*Lycopersicon esculentum*	Hubei, China	A
10	GD43	3	*Solanum melongena*	Guangdong, China	A
11	FJ1986Bd1	3	*Semen Ricini*	Fujian, China	C
12	ZJ1993Bn1	3	*Boehmeria nivea*	Zhejiang, China	A
13	GX1993Pe1	3	*Capsicum annuum*	Guangxi, China	C
14	UW148	3	*Rapistrum rugosum*	Australia	D
15	GX1993Ssp1	3	*Sesamum indicum*	Guangxi, China	C
16	GZ519	3	*Nicotiana tabacum*	Guizhou, China	A
17	GMI1000	3	*Lycopersicon esculentum*	Guyana	D
18	JS526	3	*Lycopersicon esculentum*	Jiangsu, China	A
19	GX53	3-1	*Capsicum annuum*	Guangxi, China	A
20	SC1986E4	3-1	*Solanum melongena*	Sichuan,China	C
21	FJ47	3-1	*Lycopersicon esculentum*	Fujian,China	A
22	UW76	4	*Capsicum annuum*	Armuelles, Panama	D
23	FJ2003B4	4	*Ipomoea batatas*	Fujian, China	E
24	ICPM11119	4	*Zingiber officinale*	Shandong, China	B
25	GX526	4	*Arachis hypogaea*	Guangxi, China	A
26	UW360	5	*Morus alba*	Guangdong, China	D
27	FJ4071609	5	*Arachis hypogaea*	Fujian, China	A
28	UW265	5	*Nicotiana tabacum*	Taiwan, China	D
Control strains					
No.	species				Source[a]
29	*Acidovorax avenae* subsp. *citrulli*				F
30	*Arthrobacter ilicis*				B
31	*Bacillus* sp.				A
32	*Cl. michiganensis* subsp. *sepedonicus*				G
33	*Cur. flaccumfaciens* pv. *basella* pv. nov.				A
34	*Erwinia chrysanthemi*				F
35	*Escherichia coli*				F
36	*Pantoea stewartii*				F
37	*Pseudomonas fluorescens*				F
38	*Pseudomonas putida*				F
39	*Pseudomonas syringae*				F
40	*Ralstonia pickettii*				F
41	*Rhodococcus fascians*				G
42	*Serratia* spp.				A
43	*Xanthomonas axonopodis* pv. *citri*				F

[a]Strains were contributed by the following:
A, Department of Plant Pathology, Nanjing Agricultural University, China;
B, International Collection of Micro-organisms from Plants (ICMP), Auckland, New Zealand;
C, L. Y. He, Chinese Academy of Agricultural Sciences, Beijing, China;
D, C. Allen, University of Wisconsin, Madison, Wisconsin, U.S.A.;
E, T. Lu, Fujian Academy of Agricultural Sciences, Fuzhou, China;
F, American Type Culture Collection (ATCC), U.S.A.;
G, Japan Collection of Microorganisms (JCM).

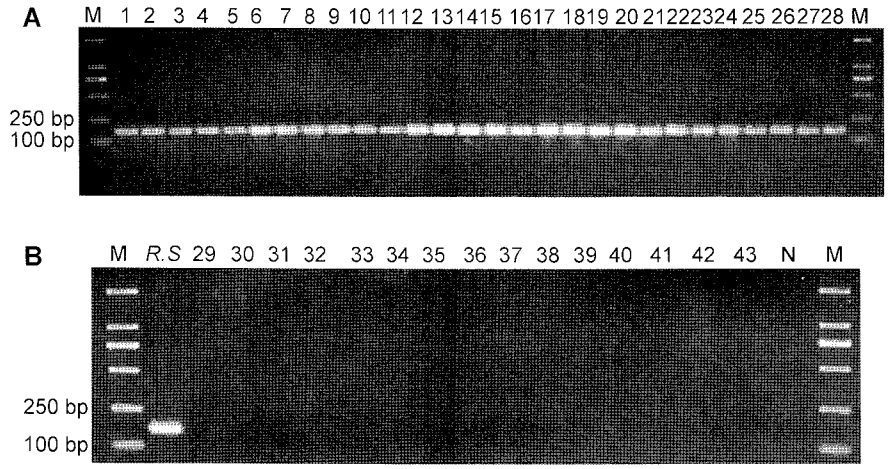

Fig. 1. PCR product banding patterns amplified from the *R. solanacearum* stains (**A**) and control strains (**B**) in this study.
Lane M: 2,000 bp marker (TaKaRa); Lane *R.S*: the model strain of *R. solanacearum* GMI1000; Lane N: negative control, addition of sterile water to the PCR mixture; Lanes 1–43 are listed in Table 1.

amplification to create a standard curve for the detection of *R. solanacearum* from tomato. This experiment included a positive control using *R. solanacearum* DNA as template, and two negative controls using DNA from healthy plant stem and sterilized water. The standard curve generated with the *R. solanacearum*-infected tomato was used for all of the tested host plants of *R. solanacearum*, including tomato, tobacco, mulberry, and ginger.

The standard curve for the detection of *R. solanacearum* from horticultural soil was established in a method similar to that mentioned above. The 10-fold serial dilutions of *R. solanacearum* (1×10^7 CFU/ml) were added and evenly mixed into the soil sample collected in a tomato field from Nanjing City of Jiangsu Province. The total DNA of each soil sample containing different dilutions of *R. solanacearum* was extracted by using the FastDNA SPIN (MP Biomedicals, LLC) for soil kit and purified by using the UNIQ gel extraction kit. In this experiment, the positive control was the DNA of *R. solanacearum*, one negative control was sterilized water, and a second negative control was the DNA of the same horticultural soil without the pathogen. The PCR protocol and reaction mixture were as described before.

The DNA templates from all of the six samples including different kinds of soils and plants were prepared as mentioned above. The quantification of these samples was conducted together with the generation of the standard curves.

Sequence Analysis

To ascertain the fragments amplified by the real-time PCR from horticultural soil and plant tissues as the right part of the upstream region of the UDP-3-*O*-acyl-GlcNAc deacetylase gene conserved only in *R. solanacearum*, the fragment amplified from the infested soil collected from Huai'an City was sequenced. The sequences were analyzed with BLAST searches (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

Results

Primer Development

The RSF/RSR primers gave a single 159-bp DNA fragment from each of 28 *R. solanacearum* strains, which represent all genetically diverse AluI types and all 6 biovars, but not from any of other control strains used in our study (Fig. 1). It indicated that the RSF/RSR primers were specific to *R.*

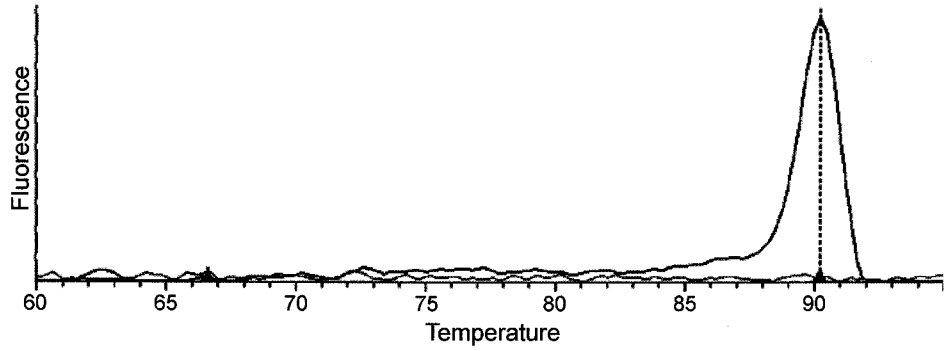

Fig. 2. The melting curve (a graph showing the rate of change in fluorescence over time as a function of temperature for each sample) is shown during the process of quantification.
A clear peak at 90.2°C is visible.

solanacearum. This 159-bp product showed a distinct T_m at 90.2°C (±0.20°C) (Fig. 2), when melting curve analysis was carried out with MJ Opticon Monitor 3 analysis software.

Specificity of Real-Time PCR in the Plants and Soil

No specific melting peak at 90.2°C was shown and no band was revealed in any of the negative control samples in real-time PCR amplifications with the primer RSF/RSR, which indicated that the pair of primers was highly specific in the detection of *R. solanacearum* in plant tissue and soil.

Sensitivity of Real-Time PCR in the Plants and Soil

The sensitivity detection experiment revealed the different signal intensities of SYBR Green I corresponding to serial dilutions of *R. solanacearum* cells (10^0–10^7 CFU/g tissue) in the plant and soil samples in real-time PCR amplifications with the primer RSF/RSR. For the detection of *R. solanacearum* from tomato stem, the limited detection concentration of *R. solanacearum* was 10^2 CFU/g tissue. Thus, the standard curve for plant samples was established using the serial Ct values that matched the pathogen concentrations from 10^2 CFU/g to 10^7 CFU/g tissue (Fig. 3).

The detection limit of real-time PCR assay was also 10^2 CFU/g in horticultural soil samples. When the concentration of *R. solanacearum* in soil was less than 10^2 CFU/g, the PCR amplification was affected by the noise of soil. According to this result, the standard curve for the soil samples was constructed using the serial Ct values corresponding to the pathogen concentrations from 10^2 CFU/g to 10^7 CFU/g tissue (Fig. 4).

Real-Time PCR Detection of *R. solanacearum* from Horticultural Soil and Plant Samples

The quantitative detection results of *R. solanacearum* from horticultural soil and plant samples and the banding patterns of PCR products of the six samples are presented in Table 2 and Fig. 5. The bacterial concentration of these ranged from 6.48×10^2 CFU/g to 5.07×10^8 CFU/g soil or plant tissue. The infested soil collected from the soybean field in Huai'an City held 8.02×10^2 CFU/g soil of *R. solanacearum*, which indicated that the pathogen had survived in soil at least 2 years although non-host plant was grown. The uninfested soil with artificially added *R. solanacearum* contained 9.77×10^3 CFU/g soil of the pathogen. The concentration of *R. solanacearum* in the stem of tomato without wilt symptom, which was sampled from the infested field in Huai'an, was 6.48×10^2 CFU/g tissue, whereas that of *R. solanacearum* reached 1.46×10^8 CFU/g tissue in the stem of tobacco with severe wilt symptom. In

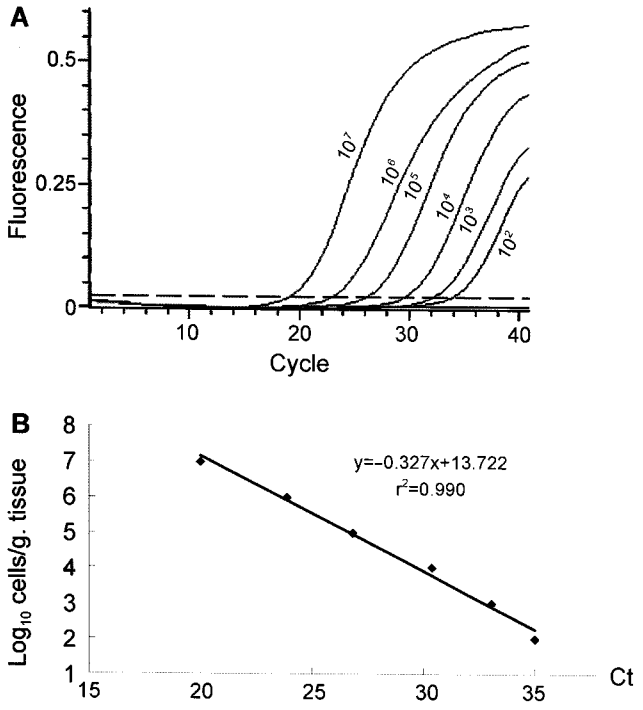

Fig. 3. The standard curve of the detection from plant tissues.
A. Kinetics of SYBR Green I fluorescence signal at different concentrations of *R. solanacearum* cells lysed by 0.5 M NaOH solution. **B.** Standard curve of the detection from plant tissues obtained by plotting the log amount of *R. solanacearum* cells (CFU/ml) against the threshold cycle of each reaction detected by real-time PCR with primer pair RSF/RSR.

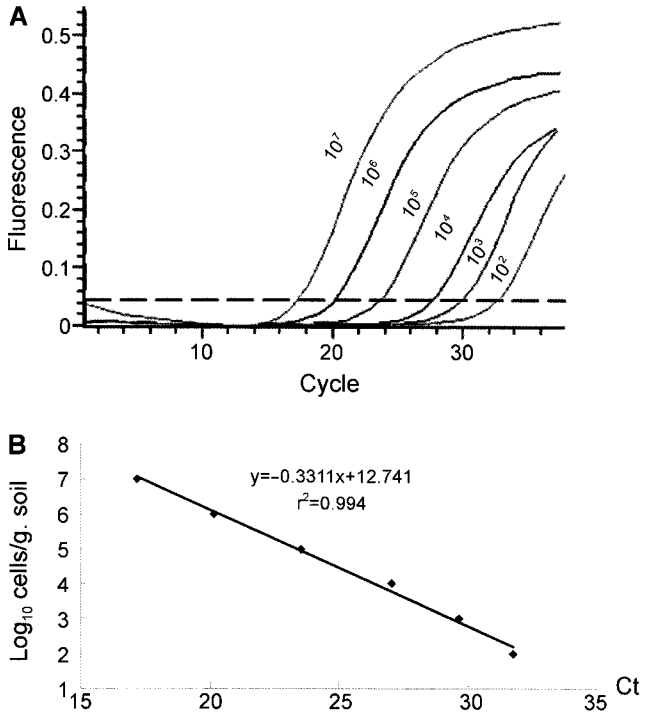

Fig. 4. The standard curve of the detection from horticultural soil.
A. Kinetics of SYBR Green I fluorescence signal at different concentrations of *R. solanacearum* cells in horticultural soil. **B.** Standard curve of the detection from horticultural soil obtained by plotting the log amount of *R. solanacearum* cells against the threshold cycle of each reaction detected by real-time PCR with primer pair RSF/RSR.

Table 2. Quantification of *R. solanacearum* DNA extracted from soil and plant tissues by real-time PCR.

Samples	Location	Natural substrates	Concentration of *R. solanacearum*
I	Huai'an, Jiangsu Province	Soil	(8.02±0.10)×10^2 CFU/g soil
II	Nanjing, Jiangsu Province	Soil	(9.77±0.05)×10^3 CFU/g soil
III	Huai'an, Jiangsu Province	Stem	(6.48±0.05)×10^2 CFU/g tissue
IV	Nanjing, Jiangsu Province	Stem	(1.46±0.02)×10^8 CFU/g tissue
V	Hangzhou, Zhejiang Province	Leaf	(4.62±0.09)×10^8 CFU/g tissue
VI	Hangzhou, Zhejiang Province	Tuber	(5.07±0.03)×10^8 CFU/g tissue

Samples I to VI were collected as follows:
I, infested soil;
II, uninfested soil with artificially added *R. solanacearum*;
III, stem of tomato without wilt symptom;
IV, stem of tobacco with severe wilt symptom;
V, uninfested leaf with artificially added *R. solanacearum*;
VI, healthy ginger tuber inoculated artificially with *R. solanacearum*.

the samples of mulberry and ginger tuber, the concentration of pathogen was 4.62×10^8 CFU/g tissue, and 5.07×10^8 CFU/g tissue, respectively.

Sequence from PCR Production

Sequencing of the fragment amplified from infested soil of the soybean field in Huai'an City by the primers indicated that this fragment was indeed the upstream region of the *R. solanacearum* UDP-3-*O*-acyl-GlcNAc deacetylase gene. The sequence was deposited into GenBank under the accession number of FJ939562.

Discussion

To lessen the huge losses *Ralstonia solanacearum* causes in subtropical and tropical areas, the rapid identification of the pathogen is the most important for disease management. To our knowledge, there was no specific and sensitive real-time PCR assay detecting directly the pathogen *R. solanacearum* from samples of soils and plants without culture and phage, although many related methods and marker genes were reported [3, 11, 12, 16, 19–21, 23, 26, 30]. In the present study, we screened 18 pairs of primers for real-time PCR, which were used by other studies [3, 12, 19, 21, 26, 30], and found that the primer pair RSF/RSR was the best in specific and sensitive detection of *R. solanacearum*. (Primers designed in our study are listed in Table 3.)

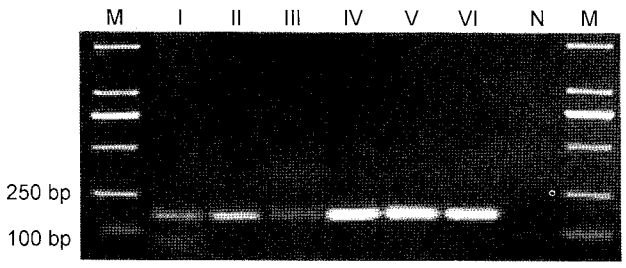

Fig. 5. PCR product banding patterns amplified from six test samples.
Lane M: 2,000 bp marker (TaKaRa); Lane N: negative control, addition of the sterilized water to the PCR mixture; Lanes I–VI are listed in Table 2.

The RSF/RSR primers can be used to specifically determine the density of *R. solanacearum* from horticultural soil. The noise in the soil could inhibit the PCR reaction or yield nonspecific PCR products. This unexpected state might result from numerous factors including some PCR inhibitors in the soil such as humic acids and the microbial diversity in soil ecosystems [13]. Microbial diversity in soil is abundant, complex, and variable. One gram of soil may harbor up to 10 billion microorganisms of possibly thousands of different species [22], but less than 1% of the microorganisms that can be observed under the microscope is cultivated and characterized [27]. Both the microbial diversity and the unculturable state caused the difficulty of specific detection of a particular microbe from the soil. However, the RSF/RSR primers in the present study might be specific to *R. solanacearum*, for all of the 15 non-target strains (Table 1) had a negative result, and the analysis in Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast) proved this specificity although we do not know much information about most of the microorganisms in the soil. Compared with immunofluorescence colony staining and plating onto SMSA [10], the number of *R. solanacearum* is more convenient, rapid, and exact. Thus, the primers RSF/RSR might be reliable for detecting *R. solanacearum* from soil and provide a method to investigate the survival of this pathogen.

Other research groups had reported that plants might begin to wilt when the *R. solanacearum* density in stems around the cotyledon reaches about 1.0×10^8 CFU/g [14, 24]. In this study, 10^2 CFU/g tissue of *R. solanacearum* could be detected from the tomato stem, which was much lower than the disease symptom needed concentration of *R. solanacearum*. Therefore, our real-time PCR detection

Table 3. Primers used in our study.

Primer	Sequence (5'-'3')	Size of product	Target gene
Rs185F	CCGAAAGCAGACTACAACC	185 bp	Endoglucanase gene
Rs185R	ACCTTCCCCTGGACAAAAT		
Rs250F	GTGCGCAATCACACCTTC	250 bp	Endoglucanase gene
Rs250R	CCTTCCCCTGGACAAAAT		
Rs119F	TCAGCTCGTCGACCACCACTC	119 bp	*hrpB*
Rs119R	GGAAGCCTTCTTCCGCTTCTT		
Rs92F	CGGTATTGCGGCACGACAC	92 bp	*hrpB*
Rs92R	CGATGAAGAAGCGGAAGAAGG		
Rs226F	AGGTCGACGCGATACAGTGA	226 bp	*hrp* conserved protein hrcu
Rs226R	ATGAAGCCAAGCCCAAGCAG		
Rs189F	GCCTTCGCTGTTCTTGTGCTC	189 bp	*hrp* conserved protein hrcu
Rs189R	GCCGCCTATCAGTCCGTCA		
Rs253F	TCAAAGCCAAGGCGTCGTATTA	253 bp	Cytochrome *c*1 signal peptide protein
Rs253R	GGACCGTTGCCGTCGTAGT		
Rs188F	GCTTTCCCGAACTCACCCCC	188 bp	Cytochrome *c*1 signal peptide protein
Rs188R	GTAGAACAAACTTGCCTGCT		
Rs114F	GCAGCCGACCAGACCAACC	114 bp	*fliC*
Rs114R	AGCCACCGAGCCGTCAAAC		
Rsol_*fliC*F[a]	GAACGCCAACG GTGCGAACT	400 bp	*fliC*
Rsol_*fliC*R	GGCGGCCTTCAGGGAGGTC		
Rs230F	AGCACCAGAAGCCGCTGTTCAT	230 bp	Popw
Rs230R	CTCCACTTTGGGCGTTCCTCC		
Rs115F	AGAACGTGTCGGAGGCGGTTT	115 bp	Popw
Rs115R	ACTTGGCTCTTGTCGGGAGC		
Rs224F	TAGTTGTTGGGGATTCATTT	224 bp	16S rRNA gene
Rs224R	CCTGTGTCCACTTTCTCTTT		
Rs207F	AATACCGCATACGACCTGA	207 bp	16SrRNA gene
Rs207R	CCATTGTCCAAAATTCCCC		
Rs199F	AGTAACTCGGCTGTTCTTT	199 bp	ITS
Rs199R	TATTCGCTTGACCCTATAA		
Rs225F	AAGTCCTACCAGACCCACC	225 bp	ITS
Rs225R	ATTGTTAAAGAACAGCCGA		
759[b]	GTCGCCGTCAACTCACTTTCC	283 bp	Upstream region of UDP-3-*O*-acyl-GlcNAc deacetylase gene
760	GTCGCCGTCAGCAATGCGGAATCG		
RSF	GTGCCTGCCTCCAAAACGACT	159 bp	Upstream region of UDP-3-*O*-acyl-GlcNAc deacetylase gene
RSR	GACGCCACCCGCATCCCTC		

[a]The primers Rsol_*fliC* F/R were reported by Schonfeld *et al.* [23].
[b]The primers 759/760 were reported by Opina *et al.* [16].
Other primer pairs used in our experiment were designed by us using Primer Premier 5.0.

method would probably become a useful tool for predicting and forecasting the *Ralstonia* wilt disease and help farmers make decisions of integrated disease management on time.

R. solanacearum is a genetically and phenotypically diverse bacterial species. It is phenotypically divided into 5 races according to host range [1, 7], and 6 biovars on the basis of their utilization of disaccharides and hexose alcohols [6]. Genetically, it is divided into 2 major groups (the "Asiaticum" and the "Americanum" divisions) according to restriction fragment length polymorphism (RFLP) analysis, 4 phylotypes, each further divided into sequevars [29]. The genetic diversity of *R. solanacearum* has been studied in strains from several countries [9]. The genetic clusters identified in these studies have close relationship with biovars. Chinese strains of *R. solanacearum* are diverse, and in our previous work, were differentiated into 13 AluI types according to the repeat domain diversity of avrBs3-like genes in this pathogen [8]. Our RSF/RSR primer pairs were extensively useful in the different strains of *R. solanacearum*, specifically to all of the 13 AluI types and 6 biovars of *R. solanacearum*, and no PCR products were amplified from the control strains. This is the first report of quantification detection of *R. solanacearum* according to the genetical diversity of the pathogen.

In conclusion, we have presented a highly sensitive and specific real-time PCR assay for the detection and quantification of *R. solanacearum* in horticultural soil and plant tissues. This might be a useful tool for restricting the spread of the pathogen by the infected plant tissues, and precisely diagnosing the *Ralstonia* wilt at the early stage of the plants in the field.

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