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PCR-based Specific Detection of *Ralstonia solanacearum* by Amplification of Cytochrome c1 Signal Peptide Sequences

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Abstract A polymerase chain reaction (PCR)-based method was developed to detect the DNA of *Ralstonia solanacearum*, the causal agent of bacterial wilt in various crop plants. One pair of primers (RALSF and RALSR), designed using cytochrome c1 signal peptide sequences specific to *R. solanacearum*, produced a PCR product of 932 bp from 13 isolates of *R. solanacearum* from several countries. The primer specificity was then tested using DNA from 21 isolates of *Ralstonia*, *Pseudomonas*, *Burkholderia*, *Xanthomonas*, and *Fusarium oxysporum* f. sp. *dianthi*. The specificity of the cytochrome c1 signal peptide sequences in *R. solanacearum* was further confirmed by a DNA-dot blot analysis. Moreover, the primer pair was able to detect the pathogen in artificially inoculated soil and tomato plants. Therefore, the present results indicate that the primer pair can be effectively used for the detection of *R. solanacearum* in soil and host plants.

Keywords: *Ralstonia solanacearum*, PCR, bacterial wilt, cytochrome c1 signal peptide, detection, diagnosis

Ralstonia solanacearum causes bacterial wilt, which is one of the most important and widely spread bacterial diseases of solanaceous crops in the tropics, subtropics, and warm temperate regions of the world. This disease has also been recorded in more than 200 species, representing over 50 plant families [11]. The recently increasing number of European sites infested with potentially cold-adapted strains of *R. solanacearum* has dramatically enhanced the threat posed to European potato crops [13, 28], emphasizing the need for efficient diagnostic tests and specific detection tools for this pathogen.

R. solanacearum has already been classified into five biovars according to carbon source utilization [9, 10] and six races based on the host range [4, 21]. The most widely used method to detect *R. solanacearum* in soil consists of streaking a soil suspension on a specific medium [7, 8]. However, the sensitivity of such a plating technique varies between soil samples, as antagonistic soil microflora often overgrow or impede the growth of *R. solanacearum* [22, 29]. Similarly, although the sensitivity of serological methods for the detection of *R. solanacearum* in plants or soil can be increased by enrichment procedures, there is also a risk that the procedure can increase populations of saprophytic bacteria in the soil suspensions, leading to false-positive reactions in an ELISA, especially when using polyclonal antibodies [5, 22].

Therefore, as an alternative, several PCR-based methods have been described for the detection of *R. solanacearum*. Seal *et al.* [27] proposed a PCR test to identify this pathogen based on its 16S rDNA sequences, whereas Boudazin *et al.* [3] developed several primers from variations within the 16S rDNA sequences between groups of strains for detection at an intraspecific level. However, both methods have limitations as regards the detection of this pathogen owing to the high degree of conservation of the ribosomal genes within the genus *Ralstonia*, leading to positive signals with related species [3, 30, 32]. DNA markers useful in PCR techniques have also been employed for the sensitive and rapid detection and identification of *R. solanacearum* strains [6, 12, 16, 25, 26, 32]. In addition, Weller *et al.* [32] developed fluorogenic PCR-based (TaqMan) assays, which proved to be sensitive for the detection of the pathogen, yet too costly and labor-intensive, considering the number of samples to be tested when compared with general PCR-based assays. Nonetheless, most of the specific primers reported in the literature lack the specificity and sensitivity for the detection of *R. solanacearum*. Accordingly, this study designed specific primers based on cytochrome c1 signal peptide sequences conserved only in *Ralstonia solanacearum* species, and then

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investigated the sensitivity and specificity of the primers for detecting *R. solanacearum* in soil and tomato plants.

MATERIALS AND METHODS

Culture Conditions and DNA Isolation

The bacterial and fungal strains were obtained from the Korean Agricultural Culture Collection (KACC), Belgian Coordinated Collections of Micro-organisms (BCCM), DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany, and American Type Culture Collection (ATCC). All the microorganisms used in this study are listed in Table 1. The conditions used to culture all the bacterial species and fungus *Fusarium oxysporum* f. sp. *dianthi* were as recommended in the *Handbook of Microbiological Media* [1]. The total DNA was extracted from the microorganisms using a genomic DNA extraction kit [Genomic-tips] supplied by QIAGEN^{T}.

Primer Design and PCR Amplification

The primers RALSF (5'-GCTCAAGGCATTCGTGTGGC-3') and RALSR (5'-GTTCATAGATCCAGGCCATC-3') were designed from the cytochrome c1 signal peptide of *Ralstonia solanacearum* isolate GMI1000 (GenBank Accession No. NP_518317; GI: 17544915), with a predicted PCR product of 932 bp. The PCR assays were performed using a PTC-225 thermocycler (MJ Research), and all the amplifications carried out in a final volume of 50 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 10 pM of each primer, and 2 units of *Taq* polymerase (Promega). The total amount

Table 1. List of bacterial and fungal isolates used in this study.

No.	Isolates	Race	Biovar	Source[a]	Geographical origin
1	*Ralstonia solanasearum*	1	1	LMG 2299	U.S.A.
2	*Ralstonia solanasearum*	1	3	LMG 2305	Egypt
3	*Ralstonia solanasearum*	1	–	KACC 11179	Japan
4	*Ralstonia solanasearum*	1	–	KACC 11178	Japan
5	*Ralstonia solanasearum*	1	3	KACC 11171	Republic of Korea
6	*Ralstonia solanasearum*	1	3	KACC 11166	Republic of Korea
7	*Ralstonia solanasearum*	3	2	KACC 10716	Republic of Korea
8	*Ralstonia solanasearum*	1	4	KACC 10704	Republic of Korea
9	*Ralstonia solanasearum*	1	3	KACC 10709	Republic of Korea
10	*Ralstonia solanasearum*	1	3	KACC 10710	Republic of Korea
11	*Ralstonia solanasearum*	3	2	KACC 10722	Republic of Korea
12	*Ralstonia solanasearum*	1	3	KACC 10714	Republic of Korea
13	*Ralstonia solanasearum*	1	3	KACC 10666	Republic of Korea
14	*Ralstonia mannitolilytica*			LMG 6866	United Kingdom
15	*Ralstonia pickettii*			LMG 5942	U.S.A.
16	*Ralstonia syzygii*			DSM 11197	China
17	*Burkholderia cenocepacia*			LMG 16656	United Kingdom
18	*Burkholderia gladioli* pv. *gladioli*			LMG 2216	U.S.A.
19	*Pseudomonas syringae* pv. *tomato*			LMG 5093	United Kingdom
20	*Pseudomonas syringae* pv. *ulmi*			LMG 2349	Yugoslavia
21	*Pseudomonas savastanoi* pv. *phaseolicola*			LMG 5008	Sweden
22	*Pseudomonas libanensis*			KACC 10809	–
23	*Pseudomonas fuscovaginae*			LMG 2158	Japan
24	*Pseudomonas coronafaciens*			LMG 5060	United Kingdom
25	*Pseudomonas citronellolis*			LMG 18378	U.S.A.
26	*Pseudomonas oryzihabitans*			LMG 7040	Japan
27	*Pseudomonas mucidolens*			LMG 2223	U.S.A.
28	*Pseudomonas graminis*			DSM 11363	Germany
29	*Pseudomonas jessenii*			ATCC 700870	–
30	*Pseudomonas lundensis*			LMG 13517	–
31	*Pseudomonas taetrolens*			LMG 2336	–
32	*Pseudomonas putida*			KACC 10272	–
33	*Xanthomonas axonopodis* pv. *citri*			KACC 10443	Republic of Korea
34	*Fusarium oxysporum* f.sp. *dianthi*			ATCC11939	–

[a]KACC, Korean Agricultural Culture Collection, Korea (http://kacc.rda.go.kr); ATCC, American Type Culture Collection, U.S.A.; LMG, Belgian Coordinated Collections of Microorganisms (BCCM), Belgium; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany.

[b]–, Unknown.

of genomic DNA from the various microorganisms added to the PCR mixture was approximately 50 ng. The reactions were run for 25 cycles, each consisting of 60 s at 94°C, 30 s at 64°C, and 60 s at 72°C, with an initial denaturation of 5 min at 94°C and final extension of 10 min at 72°C. An 8-µl aliquot of each amplified PCR product was then electrophoresed on a 1.0% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

DNA Dot-Blot Analysis

A DNA dot-blot analysis was carried out to confirm whether a fragment of the amplified cytochrome c1 signal peptide was present in the other microorganisms, including *Ralstonia* and *Burkholderi* spp., used in this study. Thus, 100-ng samples of genomic DNA isolated from the *Ralstonia* strains and other reference microorganisms were spotted onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech), and then UV cross-linked to bind the labeled probe DNA, where the PCR product from *Ralstonia solanacearum* LMG2299 was labeled as the probe with [^{32}P]dCTP using the random primed method according to the manufacturer's instructions (Ladderman). The prehybridization and hybridization were then conducted in a hybridization buffer (0.75 M NaCl, 75 mM sodium citrate, 0.5% SDS, 0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 50 µl/ml denatured salmon sperm DNA) at 65°C for 18 h. After the hybridization, the filters were washed twice (10 min each) in 2× SSC containing 0.1% SDS at room temperature and twice (15 min each) in 0.1× SSC containing 0.1% SDS at 65°C. Finally, the autoradiography was performed at −70°C using CURIX X-ray film (AGFA).

PCR Sensitivity Test

Suspensions of the *R. solanacearum* LMG2299 strain from pure cultures (adjusted to A600 nm=0.2) were serially diluted 10-fold (10^{-1}–10^{-8}) in sterile distilled water (SDW). Five-µl aliquots from each dilution series, ranging from 5×10^{8}–50 CFU/ml, were then added directly to the PCR mixture, and 5 µl of the PCR products was analyzed by electrophoresis in a 1.7% agarose gel in a 0.5× TBE buffer. To determine the CFU for the above dilutions, 100 µl of each dilution was plated onto three separate TTC [14] plates, and the plates incubated for 3 days at 28°C. All the experiments were repeated at least two times.

Detection of Bacteria in Plants and Soil

For the direct detection of *R. solanacearum* in tomato tissues, five replicate tomato seedlings were inoculated with a bacterial suspension (30 µl containing 5×10^{8} CFU/ml) of *R. solanacearum* LMG2299 based on 1-cm-long longitudinal incisions made with a sterile scalpel in the stem. The control plants were inoculated with SDW. The plants were then incubated under greenhouse conditions until symptoms developed, at which point, transverse sections (1 cm) of the stems showing bacterial wilt were cut at the soil line and macerated individually in sealed plastic bags containing 9 ml of SDW. After shaking the bags for 10 min at room temperature, the contents were serially diluted in SDW and boiled in a heating block for 5 min. The supernatant was then collected after centrifugation at 10,000 ×*g* for 1 min to precipitate the plant material, and 5 µl of the supernatant of each dilution used in the PCR. Viable counts from the samples were determined using TTC. To detect the pathogen in soil samples, 1-ml bacterial suspensions (*R. solanacearum* LMG2299) containing 5×10^{8} CFU/ml were inoculated into 9 g of a twice-autoclaved soil substrate in 50-ml conical tubes. After incubating the tubes for 2 h at room temperature, the contents were serially diluted in SDW. The supernatant was then collected as above, and 5 µl

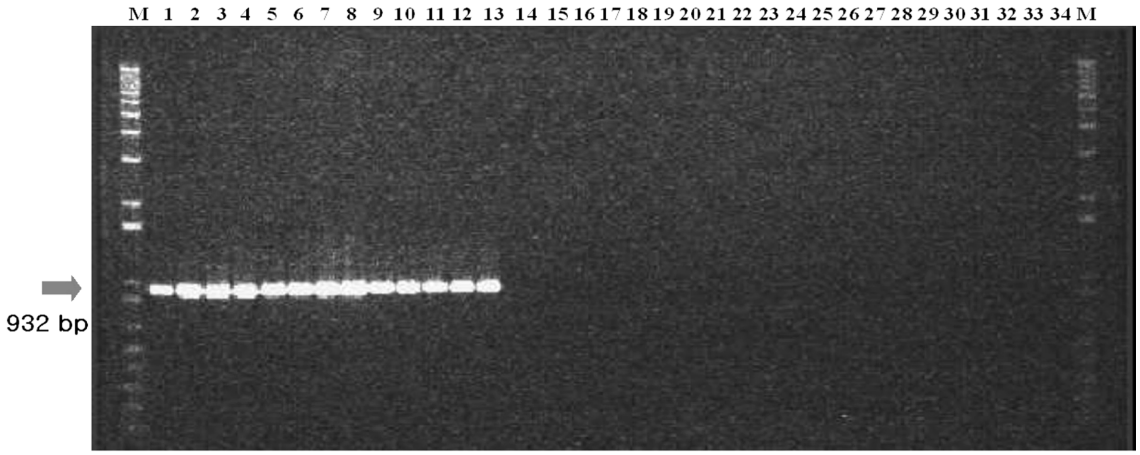

Fig. 1. PCR amplification of partial cytochrome c1 signal peptide from *Ralstonia solanacearum* using the species-specific RALSF and RALSR primer set.
Lane M, Size marker (1 kb plus DNA ladder; Gibco BRL); lanes 1–34 listed as in Table 1.

of each suspension subjected to amplification. Thereafter, the amplified DNA fragments were electrophoresed using an agarose gel and visualized following ethidium bromide staining.

RESULTS

Specificity of Primers

The RALSF and RALSR primers were tested against *Ralstonia solanacearum*. As expected, a 932 bp DNA fragment was amplified from all thirteen strains of *Ralstonia solanacearum* collected from different geographical regions (Fig. 1). To check the specificity of the primers, one fungal isolate (*Fusarium oxysporum* f. sp. *dianthi*), three other species of *Ralstonia*, and strains of *Burkholderia*, *Pseudomonas*, and *Xanthomonas* were also tested in a PCR assay using the primers RALSF and RALSR. However, none of the other *Ralstonia* species or reference microorganisms used in this study reacted with the primers (Fig. 1). Similar conditions also yielded reproducible results in a Perkin Elmer 9600 thermal cycler (Perkin Elmer International), and *Taq* polymerase enzymes supplied by different manufacturers (Promega, Takara, and Toyobo) also yielded similar PCR results.

DNA Dot-Blot Analysis

A dot-blot analysis was performed to examine whether the cytochrome c1 signal peptide sequences of the 932 bp of *R. solanacearum* shared any homology with strains of *Burkholderia, Pseudomonas*, and *Xanthomonas*, plus one fungus *Fusarium oxysporum* f. sp. *dianthi*. The probe showed a positive signal for all the *R. solanacearum* strains, suggesting that the cytochrome c1 signal peptide sequences are highly conserved in this pathogen (Fig. 2).

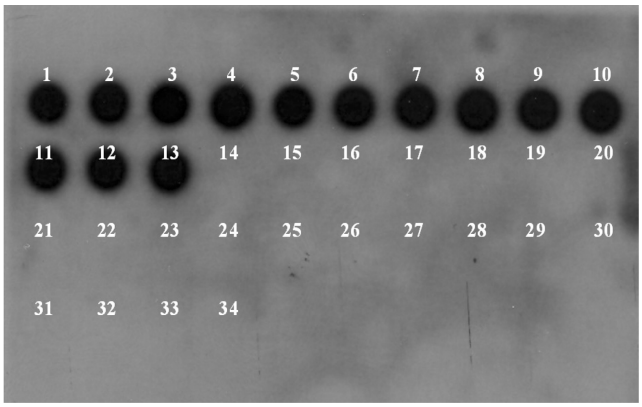

Fig. 2. DNA dot-blot analysis of cytochrome c1 signal peptide using a PCR-amplified fragment (932 bp) from *Ralstonia solanacearum* LMG2299.
Lanes 1–13, *Ralstonia solanacearum*; lanes 14 to 34, corresponding to isolates numbered in Table 1.

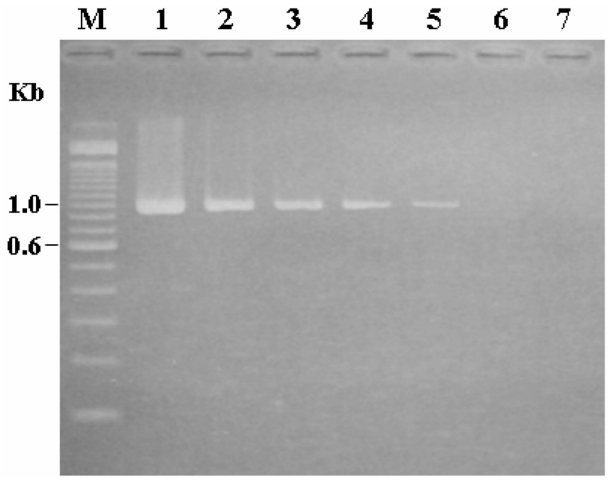

Fig. 3. Determination of the sensitivity of PCR conditions when using RALSF and RALSR primers with *Ralstonia solanacearum*.
M, Molecular size marker (100-bp ladder, Invitrogen). Lanes 1–7, dilutions of *Ralstonia solanacearum* cells ranging from 5×10^7–50 CFU/ml.

However, no signal was detected with the DNA isolated from the reference strains used in this study, indicating that the cytochrome c1 signal peptide sequences of *R. solanacearum* were absent or shared no homology with the other bacteria and fungus.

Detection of *R. solanacearum* in Soil and Tomato Plants

As the primers RALSF and RALSR were found to be specific to *R. solanacearum*, the sensitivity of the primer pair was tested using tomato tissue that had been artificially spiked with the pathogen and a soil sample inoculated with the pathogen. The primer pair was shown to amplify a 932-bp DNA fragment from both the spiked tomato tissue and soil samples, and the detection limits *in vitro* were 5×10^3 CFU/ml (Fig. 3). The species-specific PCR was also sufficiently sensitive to detect as little as 2×10^4 and 3×10^4 CFU/ml in the plant and soil samples, respectively (Figs. 4A and 4B). Healthy plant and soil samples that had been mock-inoculated with SDW as controls did not generate any visible PCR product.

DISCUSSION

The development of specific primers and DNA probes for identification and detection has already been reported for a number of plant pathogenic bacteria [15, 18–20, 23, 25 31]. Several DNA probes and PCR-based methods are also available for the detection of *R. solanacearum* [3, 25, 26, 30, 32]. However, most existing primers lead to positive signals with closely related species, despite claims of specificity and sensitivity for the detection of *R. solanacearum.*

The role of the cytochrome c1 signal peptide in microorganism pathways has not yet been fully investigated.

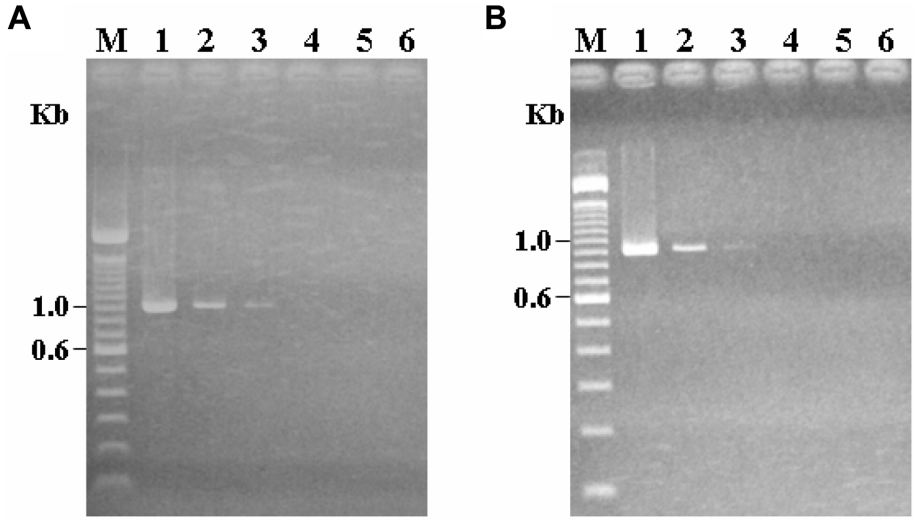

Fig. 4. PCR sensitivity assay for detection of *Ralstonia solanacearum* in artificially infected tomato plants and soil.
M. Molecular size marker (100-bp ladder, Invitrogen). **A**. PCR amplification of DNA from *R. solanacearum* in infected tomato plants. Lane 1, 2×10^6; lane 2, 2×10^5; lane 3, 2×10^4; lane 4, 2×10^3; lane 5, 2×10^2, and lane 6, 20 CFU/ml. **B**. PCR amplification of DNA from *R. solanacearum* in infected soil. Lane 1, 3×10^6; lane 2, 3×10^5; lane 3, 3×10^4; lane 4, 3×10^3; lane 5, 3×10^2, and lane 6, 30 CFU/ml.

However, Mehta *et al*. [17] recently reported that *c*-type cytochromes are specifically required for the reduction of Fe(III) oxide in *Geobacter sulfurreducens*, whereas a BLAST search revealed the cytochrome c1 signal peptide to be highly variable in different bacteria at the species level (data not shown). The nucleotide sequences of the cytochrome c1 signal peptide of *R. solanacearum* (GenBank Accession No. NP_518317, GI: 17544915) were also analyzed by a BLAST search and found to be highly variable in different bacteria at the species level (data not shown). Moreover, the primers RALSF and RALSR, which were prepared from the cytochrome c1 signal peptide sequences of *R. solanacearum*, were found to be specific for the detection of all strains of *R. solanacearum*, including different races and biovars, yet not for other phytopathogenic bacteria. (Fig. 1), suggesting that the specificity of this primer pair to *R. solanacearum* strains may be due to the unique sequence of this pathogen. Furthermore, a dot-blot analysis using the cytochrome c1 signal peptide sequences (932 bp) as a probe only showed a hybridized signal towards the genomic DNA of the *R. solanacearum* strains, and not towards any of the other phytopathogens, confirming that the cytochrome c1 signal peptide sequences were conserved in this pathogen.

It is noted that a *fliC* fragment-derived primer pair has previously been reported to be specific and sensitive for the detection of *R. solanacearum*, although it also amplified the DNA of *Pseudomonas syzygii* [25]. However, in this study, the cytochrome c1 signal peptide sequence-derived primer pair only amplified the DNA of *R. solanacearum* strains, with no exceptions, including *R. syzygii* (*P. syzygii*) (Fig. 1), suggesting that the primer pair (RALSF and RALSR) is more specific to *R. solanacearum*.

Although the successful detection of pathogens using PCR techniques essentially depends on the specificity of the primers, the PCR conditions, including the primers, template, concentration of Mg^{2+} [2], thermocyclers, and thermostable polymerase origin [24], have all been shown to affect amplification. Therefore, in this study, all these parameters were optimized to ensure the reproducibility of the amplification. As a result, consistent amplification of a 932-bp fragment from *R. solanacearum* was achieved when using different PCR machines and *Taq* polymerase enzymes supplied by various manufacturers, indicating the usefulness of the RALSF and RALSR primers for the specific detection of *R. solanacearum*.

Furthermore, the current results clearly demonstrated the sensitivity of the primer pair (RALSF and RALSR) for the detection of *R. solanacearum* in artificially infected soil, tomato stems, and pure cultures. When cell suspensions or tissue extracts are directly used for a PCR, the appearance of unspecific amplification bands is a common problem, which hampers the interpretation of the results. However, no spurious bands were observed in any of the samples analyzed using the RALSF and RALSR primers, indicating that this primer set is highly specific to *R. solanacearum* and reliable for the detection and diagnosis of this pathogen.

Acknowledgment

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