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The design of specific primers for the detection of *Ralstonia solanacearum* in soil samples by polymerase chain reaction

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Abstract. A 0.7-kb DNA fragment, amplified by the randomly amplified polymorphic DNA (RAPD) method from *Ralstonia solanacearum* total DNA, was cloned and evaluated as a specific DNA probe. This 0.7-kb DNA fragment hybridized to a 2.7-kb *Eco*RI fragment in the *Eco*RI-digested total DNA of *R. solanacearum*. The 2.7-kb *Eco*RI fragment was also cloned and hybridized only to *R. solanacearum* but not to other *Pseudomonas* spp., pathovars of *Xanthomonas campestris*, and *Erwinia* spp. tested. The DNA sequence of this 2.7-kb fragment was obtained and used to design specific oligonucleotide primers for polymerase chain reaction (PCR) amplification. The primers amplified the same 1.1-kb PCR product from all *R. solanacearum* strains tested and failed to amplify DNA from any other plant pathogenic bacterial strains tested. DNA isolated from several saprophytic bacteria did not produce any PCR products with these primers. This specific PCR for *R. solanacearum* was also performed from colonies grown on BG medium with similar results. The sensitivity of the PCR assay using the specific primers was about 20 cells. The PCR assay was used to detect *R. solanacearum* in soil using these primer sets. No PCR product could be found when soil extract containing *R. solanacearum* was used directly in the assay. DNA extraction from soil was needed for the success of PCR assay. A simple method for DNA extraction from soil for PCR assay was developed and can hasten the detection of *R. solanacearum* in soil.

Keywords: *Erwinia*; Polymerase chain reaction; *Pseudomonas*; *Ralstonia solanacearum*; *Xanthomonas*.

Introduction

Ralstonia solanacearum (synonym *Pseudomonas solanacearum* E. F. Smith) (Yabuuchi et al., 1995) causes bacterial wilt, one of the most important and widespread bacterial diseases of crops in the world. This pathogen has been recorded on several hundred species representing 44 families of plants (Buddenhagen and Kelman, 1964; Hayward, 1991). In Taiwan, bacterial wilt has been reported on tomato, tobacco, potato, sweet pepper, eggplant, bird-of-paradise (Yang et al., 1980), perilla (Perilla crispa) (Hong et al., 1990), radish (Lin et al., 1994), anthurium plants (Su and Leu, 1995), and eustoma (Chao et al., 1995). *Ralstonia solanacearum* can be spread within and between countries by soil, by water, and by latently infected planting materials (Ciampi et al., 1980; Hayward, 1991). Accordingly, rapid and highly sensitive detection methods of *R. solanacearum* are required for quarantine to reduce field losses and limit the spread of bacterial wilt (Seal et al., 1992a, 1993; Seal, 1995).

The success of quarantine procedures relies on the use of rapid and sensitive detection techniques. Traditional methods for detecting *R. solanacearum* depend on a series of biochemical tests on purified colonies. This identification procedure is too time consuming for quarantine and other diagnostic laboratory purposes. Nucleic acid probes and polymerase chain reaction (PCR), on the other hand, have been widely reported as tests that can be performed without culturing. Diagnostic tests based on PCR amplification of plant pathogens from environmental samples have shown that rapid and specific detection of very low numbers of the pathogen can be achieved (Seal et al., 1992a, 1993; Seal, 1995).

Development of a DNA probe specific for *R. solanacearum* is complicated by the genetic diversity of this species. *Ralstonia solanacearum* strains represent a heterogeneous group that has been subdivided informally into five races on the basis of host range (Buddenhagen et al., 1962; Buddenhagen, 1986), or five biovars based on the ability to oxidize certain disaccharides and hexose alcohols (Hayward, 1964; He et al., 1983). In addition, there is a phylogenetic dichotomy in *R. solanacearum*, which can be separated into two divisions based on the analysis of restriction fragment length polymorphism patterns (Cook et al., 1989), PCR amplification with tRNA consensus primers (Seal et al., 1992b), and rRNA sequences (Li et al., 1993; Seal et al., 1993; Seal et al., 1999; Taghavi et al., 1996). Division I contains all members of race 1 biovars 3, 4 and 5, and division II contains all members of race 1 biovar 1 and races 2 and 3. Strains of *R. solanacearum* in Taiwan are all race 1, and most are biovar 3 though some are biovar 4 (Hsu, 1991). These strains are all assigned to

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division I. Although DNA fragments specific for *R. solanacearum* and even race 3 strains have been cloned by subtractive hybridization, they did not specifically hybridize to all *R. solanacearum* strains tested (Cook and Sequeira, 1991; Seal et al., 1992a; Opina et al., 1997). For effective quarantine and detection measures against *R. solanacearum*, it is necessary to develop a specific and sensitive diagnostic test for *R. solanacearum* strains in Taiwan. We describe here the use of the randomly amplified polymorphic DNA (RAPD) technique to identify an *R. solanacearum*-specific DNA fragment. This DNA fragment was subcloned, the DNA sequences were determined, and specific oligonucleotide primers were designed for detection of *R. solanacearum* by PCR. Furthermore, a simple method for DNA extraction from soil for PCR assay was developed to detect the existence of *R. solanacearum* in soils.

Materials and Methods

Bacterial Strains and Culture Conditions

The strains of *R. solanacearum*, *Pseudomonas syringae*, *Xanthomonas*, and *Erwinia* used in this study, their pathovar designations, and sources are listed in Table 1. *Ralstonia solanacearum* was cultured in BG media (Boucher et al., 1985) at 30°C. *Escherichia coli* DH5*a* (BRL Life Technologies, Inc., Gaithersburg, MD), *X. campestris*, *Erwinia* spp. and *P. syringae* were cultured in Luria-Bertani agar or broth medium (Sambrook et al., 1989) at 37°C for *E. coli* and at 28°C for the other species. Ampicillin (50 μg/ml) was added as necessary to maintain selection of the resistance marker in pBluescript SK(+) (Stratagene Co., La Jolla, CA).

General DNA Manipulations

Mini-scale preparations of *E. coli* plasmid DNA, total genomic DNA isolation of plant pathogenic bacteria, restriction endonuclease treatments, DNA ligation, transformation, and agarose gel electrophoresis were done as described by Sambrook et al. (1989). PCR-amplified DNA fragments used as probes were recovered from agarose by using the QIAEX II Gel Extraction Kit (QIAGEN Inc. Chatsworth, CA) and labeled with digoxigenin-11-dUTP (DIG) using a PCR DIG probe synthesis kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The PCR for preparation of DIG-labeled DNA probes was performed in an air thermal cycler (Rapidcycler, Idaho Technology, ID) programmed for denaturation at 94°C for 2 min and then for 35 cycles of 0 sec at 94°C, 0 sec at 50°C, and 35 sec at 72°C. Prehybridization, hybridization, and washing for Southern hybridization with DIG-labeled probes were performed at 68°C according to the

Table 1. Bacterial strains and hybridization results of the 2.7 kb *Eco*RI fragment of *R. solanacearum* to the bacteria.

Positive species and strains[a]
- *R. solanacearum* strains[b]:
  - from tomato: PS21; PS-S4; PS-S32; PS-S80; PS68; PS72; PS95.
  - from tobacco: PS31; PS48.
  - from potato: PS60.
  - from sweet pepper: PS87; PS96.
  - from eggplant: PS99; PS100.
  - from anthurium: PS-AU003.
  - from New Zealand flax: PS-PDN002; PS-PDN003.
  - from cat-tail willow: PS-CTW009.
  - from eustoma: PS-ES001; PS-ES008.
  - from rose: PS-RO005; PS-RO006.

Negative species and strains[a]
- Pseudomonas species and strains[c]
  - *P. cepacia* PC22; *P. gladioli* UCBPP550; *P. syringae* pv. *apii* PA102; *P. syringae* pv. *tabaci* PT124; *P. syringae* pv. *eriobotryae* UCBPP258; *P. syringae* pv. *atrofaciens* UCBPP260; *P. syringae* pv. *coronafaciens* UCBPP470; *P. syringae* pv. *glycinea* UCBPP527.
- *X. campestris* pathovar[c]
  - *X. campestris* pv. *betlicola* XB104; *X. campestris* pv. *campestris* XCC1-1; *X. campestris* pv. *dieffenbachiae* XD113; *X. campestris* pv. *oryzicola* XO111; *X. campestris* pv. *pelargonii* XP15; *X. campestris* pv. *phlei* XP3-5; *X. campestris* pv. *punicae* XP177; *X. campestris* pv. *spermacoces* XS8-9; *X. campestris* pv. *vesicatoria* XV123.
- *Erwinia* spp.[c]
  - *E. amylovora* EA169; *E. carotovora* subsp. *atroseptica* UCBPP149; *E. carotovora* subsp. *carotovora* Erc1; *E. cypripedii* EC155; *E. quercina* EQ101; *E. rhapontici* ER102; *E. rubrifaciens* ER103.

[a]The positive strains have hybridization signal with 2.7-kb *Eco*RI DNA fragment in Southern blot hybridization. The negative strains have no signal. The hybridization method is described in Materials and Methods.

[b]The strains were obtained from Department of Plant Pathology, National Chung Hsing University, Asian Vegetable Research and Development Center and Department of Pesticide Application, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taiwan.

[c]The strains were obtained from the International Collection of Phytopathogenic Bacteria, University of California, Berkeley, and American Type Culture Collection, Rockville, MD, USA.

manufacturer's protocol. Double-stranded sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) adapted for the AutoRead Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) with standard universal forward and reverse M13 primers.

Primer and PCR Conditions

For RAPD analysis, PCR amplifications were carried out in 10-µl volumes which contained 10 ng of genomic DNA, 3 mM $MgCl_2$, 0.5 µM primer, 0.4 U of Taq DNA polymerase (DyNAzyme II, Finnzymes Oy, Finland), 200 µM (each) deoxynucleoside triphosphate (dNTP) in 50 mM Tris HCl (pH 8.3), and 500 µg/ml BSA. Amplification was performed in an air thermal cycler programmed for two cycles of 70 sec at 94°C, 17 sec at 42°C, and 80 sec at 72°C; 35 cycles of 10 sec at 94°C, 17 sec at 42°C, and 80 sec at 72°C; and a final extension for 4 min at 72°C. Fifteen different arbitrary primers from 15-22 bases in length were used. Oligonucleotide primers were synthesized with a DNA synthesizer by the Agricultural Biotechnology Laboratories, National Chung Hsing University, Taiwan. Amplified DNAs were detected by electrophoresis in 0.8% agarose gels.

For specific amplification of *R. solanacearum*, two oligonucleotide primers, BP4-R and BP4-L, were designed from the nucleotide sequence of 2.7-kb *Eco*RI fragment cloned from *R. solanacearum* PS68. BP4-R (5'-GACGACATCATTTCCACCGGGCG-3') and BP4-L (5'-GGGTGAGATCGATTGTCTCCTTG-3') delineated a 1,102-bp fragment. DNA was amplified in 10-µl of reaction mixture prepared as described above. PCR condition was programmed for denaturation at 94°C for 2 min and then for 35 cycles of 0 sec at 94°C, 0 sec at 50°C, and 70 sec at 74°C. To determine the minimum number of *R. solanacearum* that could be detected by PCR, 10-fold serial dilutions of PS68 strain were made. Two µl aliquots from each tube of the dilution series were added directly to the PCR mixture. Aliquots from undiluted and serial dilutions of cultures were spread on BG plate to count the bacteria, and colony-forming units (cfu) was determined.

Soil and Soil Inoculation

Soil samples were collected from Yang-Ming mountain and Hsin-Chuang agriculture areas in northern Taiwan. Surface soil samples (depth, 0 to 15 cm) were collected, sieved (mesh size 2 mm), and stored under field-moist conditions at 4°C. The soil samples had pH range from 6.5 to 8.1. Subsamples (1 g) were seeded with 0.1-ml decimal dilutions of *R. solanacearum* culture which originally contained 10^7 cfu/ml. The inoculated soil samples were incubated for one hour at room temperature to equilibrate and allow for binding of the bacterial cells to the soil colloids.

Extraction of DNA from Soil for PCR Reaction

All soil samples were quantified as wet weight throughout the experiments. Total DNA was extracted from soil samples by a rapid method modified from the DNA isolation method developed by Chowdhury (1991). Inoculated soil samples (0.2 g) were suspended in 0.5 ml of distilled water by vortexing and allowed to stand for 5 min. The supernatant was transferred to a new microfuge tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:l, v/v/v; pH 7.6) was added, after which they were mixed by vortexing at the maximum speed for 1 min and centrifuged at 16,000 *g* for 10 min. After the aqueous phase was transferred to a new tube, 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol were added. The solution was mixed well and centrifuged at 16,000 *g* for 15 min. DNA pellets were washed with 70% ethanol twice, vacuum dried, and resuspended in 20 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer. Two µl of this DNA solution were taken for further PCR analysis.

Results

RAPD Analysis and Selection of a DNA Fragment Specific to R. solanacearum

To identify primers that generated RAPD patterns characteristic of *R. solanacearum*, 15 different primers were tested with DNA extracted from *R. solanacearum* PS68 and several pseudomonads. The amplification pattern obtained with primer R-424 on the DNA extracts of *R. solanacearum* PS68 was reproducible and revealed one major product of 0.7-kb. The sizes of the products amplified from pseudomonads ranged from 0.3 to 2.8 kb (Figure 1). All of the *R. solanacearum* strains tested by RAPD with R-424 primer exhibited the same pattern (data not shown). The 0.7-kb DNA fragment was eluted from agarose gel, la-

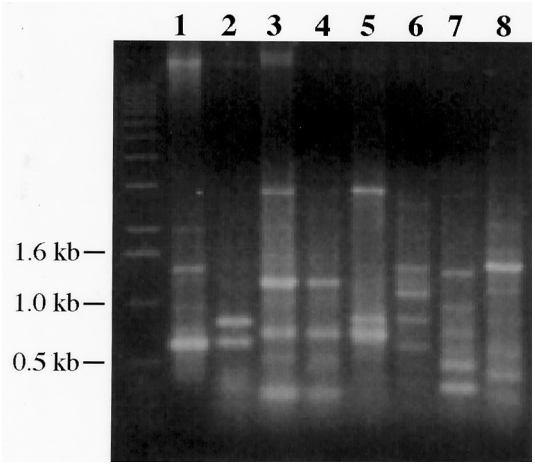

Figure 1. Band patterns obtained after RAPD amplifications of *R. solanacearum* and other plant pathogenic bacteria strains with the oligonucleotide R-424. A 0.7-kb DNA fragment was amplified with DNA from *R. solanacearum*. Lanes: 1, *R. solanacearum* PS68; 2, *Pseudomonas cepacia* PC22; 3, *P. syringae* pv. *apii* PA102; 4, *P. syringae* pv. *tabaci* PT124; 5, *P. syringae* pv. *eriobotryae* UCBPP258; 6, *P. syringae* pv. *atrofaciens* UCBPP260; 7, *P. syringae* pv. *glycinea* UCBPP527; 8, *P. gladioli* UCBPP550. The size of the band is indicated on the left.

beled with DIG by PCR with the R-424 primer, and used as a probe against *Eco*RI-digested total DNAs of *R. solanacearum* and several pseudomonads. The result showed that the 0.7-kb DNA fragment hybridized to a 2.7-kb *Eco*RI fragment in the genomic DNA of *R. solanacearum* PS68, but not with those of any pseudomonads tested (Figure 2).

To isolate and identify the 2.7-kb *Eco*RI fragment responsible for this specific hybridization, the region of an agarose gel containing 2.7-kb fragments of *Eco*RI-digested genomic DNA of *R. solanacearum* PS68 was excised. The DNAs were recovered from the gel, ligated to *Eco*RI-digested pBluescriptSK(+), and used to transform competent *E. coli* DH5*a* cells. Plasmid DNAs were isolated from 80 transformants and, after digestion with *Eco*RI and agarose gel electrophoresis, were hybridized with DIG-labeled 0.7-kb fragment. Three positive clones were selected. The 2.7-kb *Eco*RI fragment in one of the positive clones was eluted, labeled by the random primer method, and used to probe *Eco*RI-digested genomic DNAs from *R. solanacearum* PS68 and several pseudomonads. The probe hybridized only to *R. solanacearum* PS68 but not to the pseudomonads tested. The same probe was also used to hybridize genomic DNAs from other plant pathogenic bacteria, such as pathovars of *Xanthomonas campestris*, *Erwinia* spp., and several strains of soil saprophytic bacteria, and no hybridization signal was found (Table 1). Because longer DNA fragments contain more genetic information, the 2.7-kb fragment was used instead of the 0.7-kb fragment in further analysis.

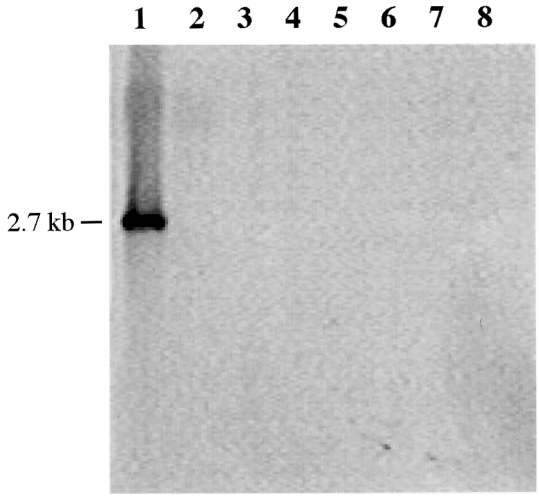

Figure 2. Hybridization of the 0.7-kb fragment amplified from *R. solanacearum* with R-424 to *Eco*RI-digested total genomic DNA of *R. solanacearum* and other plant pathogenic bacteria strains. DNA (0.5 μg) was loaded into each lane, electrophoresed in 0.8% agarose, and hybridized with the digoxigenin-11-dUTP-labeled probe. Lanes: 1, *R. solanacearum* PS68; 2, *P. cepacia* PC22; 3, *P. syringae* pv. *apii* PA102; 4, *P. syringae* pv. *tabaci* PT124; 5, *P. syringae* pv. *eriobotryae* UCBPP258; 6, *P. syringae* pv. *atrofaciens* UCBPP260; 7, *P. syringae* pv. *glycinea* UCBPP527; 8, *P. gladioli* UCBPP550. The size of the band is indicated on the left.

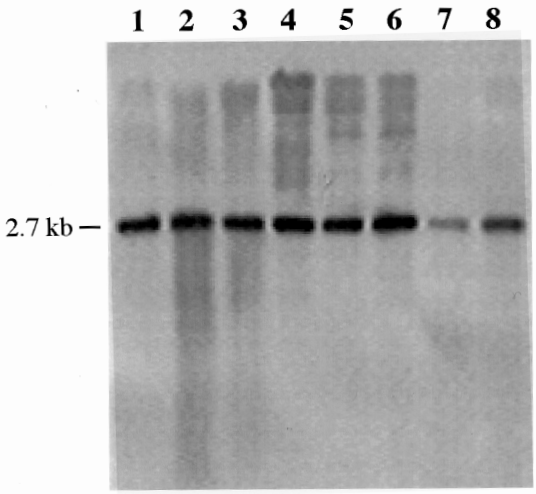

Figure 3. Hybridization of the 2.7-kb *Eco*RI fragment to *Eco*RI-digested total genomic DNA of different strains of *R. solanacearum*. DNA (0.5 μg) was loaded into each lane, electrophoresed in 0.8% agarose, and hybridized with the digoxigenin-11-dUTP-labeled probe. Lanes: 1, *R. solanacearum* PS-31; 2, PS-60; 3, PS-87; 4, PS-AU003; 5, PS-PDN002; 6, PS-CTW009; 7, PS-ES001; 8, PS-RO005. The size of the band is indicated on the left.

To determine if the specificity of the 2.7-kb *Eco*RI fragment for *R. solanacearum* PS68 was indicative of a broader specificity for all strains isolated in Taiwan, the 2.7-kb fragment was used as a hybridization probe against total DNA extracted from all strains of *R. solanacearum* shown in Table 1. It was found that the 2.7-kb fragment hybridized with genomic DNAs of all *R. solanacearum* strains tested. Representative results are shown in Figure 3.

Specific Primers for Detection of R. solanacearum by PCR

The positions of restriction endonuclease sites and DNA sequence of the 2.7-kb *Eco*RI fragment were determined. Based on these DNA sequences, two oligonucleotides (BP4-R and BP4-L) were synthesized and used for subsequent PCR amplification tests. To determine the specificity of these primers, PCRs were carried out with DNA of all of the strains listed in Table 1. The PCR amplification using BP4-R and BP4-L primers generated an approximately 1.1 kb amplified DNA fragment for all *R. solanacearum* strains tested. No amplification was observed with any other *Pseudomonas*, *Xanthomonas*, or *Erwinia* strains tested.

Identical results were obtained when this specific PCR was performed with *R. solanacearum* PS68 colonies grown on BG medium. To determine the sensitivity of detection of the PCR assay with primers BP4-R and BP4-L, 2-μl samples of serial dilutions of *R. solanacearum* PS68 culture were used as template for the PCR. The tests were repeated six times, and the sensitivity of detection was determined as 10^4 cfu per ml of bacterial suspension on the

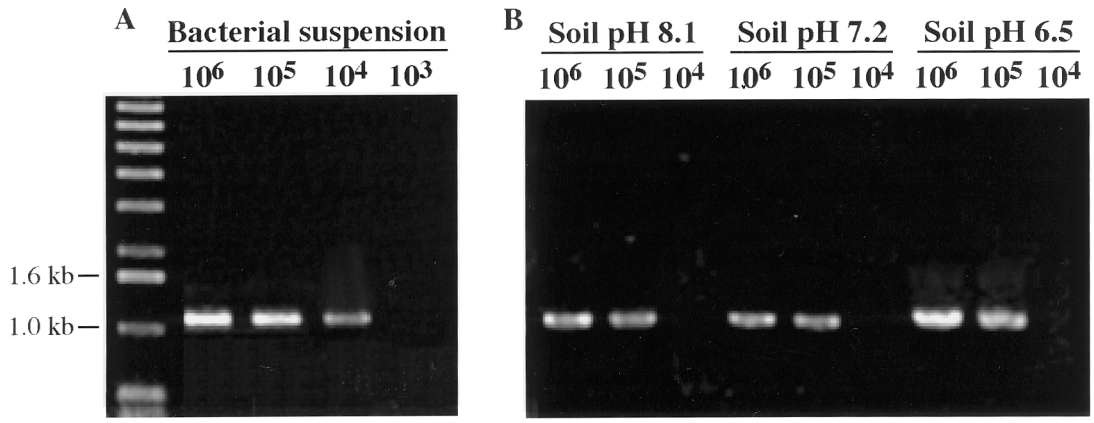

Figure 4. Detection of *R. solanacearum* by PCR-specific reaction with BP4-R and BP4-L primers on (A) suspensions of *R. solanacearum* strain PS68 (cfu/ml) and on (B) crude DNA extracted from three soil samples with pH from 6.5 to 8.1 inoculated with different amounts of PS68 (cfu/g). Molecular sizes are given in kb.

ethidium bromide-stained gel (Figure 4A). Since only 2 µl of bacterial suspension were used for PCR, this method was able to detect 20 cells.

PCR assay for the Detection of R. solanacearum in Soil

No PCR product could be found when soil extract containing *R. solanacearum* was used directly in the PCR assay. DNA extraction from soil was needed for the success of PCR assay. The quality and quantity of DNA, obtained by using our simple DNA extraction method from soil, can be used for PCR analysis. Following the direct DNA extraction from each inoculated soil, a 2-µl aliquot from 20 µl of crude DNA was used as a template for PCR reaction, resulting in an amplification of 1.1-kb fragment by BP4-R and BP4-L primers as shown in Figure 4B. The sensitivity of detection was determined as 10^5 cfu per gram of soil on the ethidium bromide-stained gel. However, since only 0.2 gram of soil sample and 0.1 volume of the DNA extraction solution was used for PCR, this method was actually able to detect 2×10^3 cells. No amplification of target sequence was observed in uninoculated soils.

Discussion

DNA-based methods have provided powerful tools to identify and detect microorganisms with high sensitivity and specificity. The RAPD procedure was used to find a specific 0.7-kb DNA fragment unique to *R. solanacearum*. By using this 0.7-kb fragment as a probe, a 2.7-kb *Eco*RI fragment from *R. solanacearum* PS68 was identified and cloned. The usefulness of this 2.7-kb *Eco*RI fragment for diagnostic and detection tests may depend on the frequency with which *R. solanacearum* strains have homology to the fragment. Since strains of division II of *R. solanacearum*—including race 1 biovar 1 and races 2 and 3—have not yet been found in Taiwan, they were not tested in this study. Nevertheless, the tested strains of *R. solanacearum* were isolated from 10 different host plants, and were therefore considered to be representative samples of this species in Taiwan. It was found that the 2.7-kb fragment hybridized with genomic DNAs of all *R. solanacearum* strains tested. An intriguing question with respect to this specific DNA concerns its function. Analysis of the 2.7-kb *Eco*RI DNA fragment revealed no significant relatedness to any known sequences present in the EMBL/GenBank Data Library. The DNA fragment was found to contain at least one potential open reading frame which specifies a protein of 472 amino acids (data not shown). This potential product also showed no significant similarity to any known proteins in the protein data banks. Furthermore, since it was possible that pathogen-specific DNA might encode information required for disease, the DNA region is being deleted to assess the effect of the deletion on pathogenicity or virulence of *R. solanacearum*.

The PCR primers designed in this study were shown to be specific to *R. solanacearum* and produced a more easily detected PCR product than the previous PCR primers used. By constructing specific primers BP4-R and BP4-L based on the sequence of 2.7-kb *Eco*RI DNA fragment, *R. solanacearum* could be detected between 10 and 50 cells in suspensions, without the need for prior enrichment or cultivation. Cook and Sequeira (1991) utilized a subtraction hybridization procedure to enrich *R. solanacearum* race 3-specific sequences and obtained a probe that reacted with all race 3 strains but with only 5 of 90 non-race 3 strains tested. This probe can not be used in Taiwan because strains of *R. solanacearum* isolated in Taiwan are all race 1. Two specific primer sets, PS96-H/PS96-I and 759/760, have been designed for detection of *R. solanacearum* (Opina et al., 1997; Seal et al., 1992a). These primer sets, however, amplified 148-bp and 281-bp PCR products from *R. solanacearum* DNA, respectively, which are mobilized together with oligonucleotide primers and bacterial RNAs and have to be electrophoresed on a high

percentage of agarose gel (e.g. 1.5-2.0%). The PCR primers BP4-R and BP4-L designed in this study yielded a 1.1-kb product that can be easily visualized on a normal 0.8-1.0% agarose gel.

Soil is a vehicle for the spread of *R. solanacearum*, and a high level of wilting occurred in soils previously planted with susceptible plant cultivars (Hayward, 1991). For effective quarantine measures and disease control, it is important to develop a specific, rapid, and sensitive detection method for *R. solanacearum* in soil. The application of PCR in soil analyses is limited by the labor-intensive extraction of total DNA from soil. In this paper we modified a DNA isolation method to isolate total DNA from soil. The method was originally developed by Chowdbury (1991) for a rapid plasmid isolation, but it can also be used to extract chromosomal DNA from cells. Isopropanol was used to precipitate DNA instead of ethanol, because it is more selective for DNA during precipitation (Sambrook et al., 1989) and can remove PCR inhibitors (Hänni et al., 1995). The DNA extracted from soil by this simple method can be directly used for PCR analysis, although the sensitivity on DNA extracted from soil samples (2×10^3 cells) was lower than that on bacterial suspension (20 cells). The decreased sensitivity could be attributed to binding of the bacterial cells to the soil colloids and PCR-interfering compounds present in the soil (Tsai and Olson, 1992). The sensitivity of PCR for soil samples may be improved by using selective enrichment methods (Brook et al., 1997; Ito et al., 1998). Selective enrichment medium can be added into soil suspension. After incubation to increase bacterial cell number, total DNA can be easily extracted by this method for further PCR analysis.

In conclusion, a DNA probe and a PCR specific to *R. solanacearum* are described here. The use of this PCR and the simple DNA extraction method from soil offer a rapid method for the identification and detection of *R. solanacearum* and can be employed in analyzing and monitoring plant materials and soil where *R. solanacearum* is usually implicated.

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以聚合酵素連鎖反應檢測土壤內青枯病菌的專一性引子的設計

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對青枯病菌的基因組核酸（genomic DNA）以任意放大多型性核酸（RAPD）技術，進行聚合酵素連鎖反應（PCR）的分析，發現可擴增出一段 0.7-kb 的 PCR 產物。以此 PCR 產物做為探針，與經 *Eco*RI 酵素處理的青枯病菌的基因組核酸，進行南方氏雜合反應，可在 2.7-kb 處有雜合訊息。將此 2.7-kb *Eco*RI 核酸片段選殖出後，再以此片段做為探針進行雜合反應，發現只有青枯病菌的基因組核酸可產生雜合訊息，而其他植物病原細菌如 *Pseudomonas* spp.、*Xanthomonas campestris* 病原小種、*Erwinia* spp. 等均無訊息產生。將此 2.7-kb *Eco*RI 核酸片段經核酸定序，並依該序列設計出一組 PCR 引子，此組引子對青枯病菌具有專一性，可擴增出 1.1-kb 的 PCR 產物，且 PCR 反應的敏感度可以達到 20 細胞。此 PCR 反應亦用於檢測土壤內的青枯病菌，若直接以含有青枯病菌的土壤萃取液進行 PCR反應，發現無任何 PCR產物。若從含有青枯病菌的土壤抽取核酸後再進行 PCR 反應，則可擴增出 1.1-kb 的 PCR 產物。為加速對土壤內青枯病菌的檢測工作，已研發出一個從土壤抽取核酸以進行 PCR反應的簡易方法。

關鍵詞：軟腐病菌；聚合酵素連鎖反應；假單胞菌；青枯病菌；黃原桿菌。