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A Novel Insertion Sequence, IS*Rso19*, Isolated from *Ralstonia solanacearum* and Its Application to Race Differentiation

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ABSTRACT

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A new insertion sequence (IS), IS*Rso19*, was isolated and characterized from a *Ralstonia solanacearum* race 2 strain JS775. Sequence analysis indicated that the IS is closely related to the members of IS21 family, but the extent of nucleotide sequence identity in 5'- and 3'-noncoding regions between IS*Rso19* and other members of IS*21* family is only about 43%. Nucleotide sequence of these regions was used to design specific oligonucleotide primers for detection of race 2 strains by PCR. The PCR amplified a specific DNA fragment for all *R. solanacearum* race 2 strains tested, and no amplification was observed with *R. solanacearum* race 1 strains and some other plant pathogenic bacteria tested. Accordingly, the IS*Rso19* isolated is a specific marker for *R. solanacearum* race 2 strains. In addition, a multiplex PCR assay that comprised specific primers targeted to IS*Rso19* and IS*1405*, was established to detect and differentiate *R. solanacearum* race 1 and 2 strains.

Key words: Insertion sequence, IS*Rso19*, IS*1405*, *Ralstonia solanacearum*

INTRODUCTION

Bacterial wilt of triploid banana (Moko disease) and *Heliconia* is caused by race 2 of *Ralstonia solanacearum*. Its effect on bananas is devastating as it causes serious wilt of plants, eventually leading to death [2,3]. The Moko disease has not been recorded in Taiwan. Importation of banana plant parts and whole plants is prohibited from countries where Moko disease has been reported. However, such regulation is not applied to ornamental *Musa* and *Heliconia* plants. These ornamental plants may serve as a host and has the potential to carry the Moko pathogen [3]. Thus, a sensitive molecular detection method is needed for effective quarantine measures to prevent the introduction and spread of Moko pathogen.

A simple subtraction hybridization method was employed to isolate specific DNA fragments from DNA of *R. solanacearum* race 2 strain. A cloned DNA fragment had homology with a putative transposase. The region flanking the cloned DNA fragments was isolated, and sequence analysis showed that the region contains a novel insertion sequence, designated IS*Rso19*. IS*Rso19* was used as a specific marker for detection of *R. solanacearum* race 2 strains. In addition, a multiplex PCR assay, which comprised specific primers targeted to IS*Rso19* and IS*1405* [6], was established to differentiate *R. solanacearum* race 1 and 2 strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The strains of *R. solanacearum*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* used in this study are listed in Table 1. Whenever an *Escherichia coli* host was necessary, strain DH5α (Gibco-BRL Life Technologies, Inc., Gaithersburg, MD) was used. *R. solanacearum* was cultured in BG media [1] at 30℃. *E. coli* DH5α, *Xanthomonas* spp., *Erwinia* spp., and *Pseudomonas* spp. were cultured in Luria-Bertani medium [9] at 37℃ for *E. coli* and at 28℃ for the other species. Ampicillin (50 μg ml^{-1}) was added as necessary to maintain selection of the resistance marker in pBluescript SK(+) (Stratagene Co., La Jolla, CA).

Subtractive hybridization

Subtractive hybridization was carried out according to the method described by Seal *et al.* [11]. *R. solanacearum* race 1 PS68 strain was used as the driver strain, and 1 μg of

Table 1. Bacterial strains tested

R. solanacearum strains:	
race 1 strains [1]	
tomato: PS21, PS68, PSS4, PSS180, PSS81; tobacco: PS31; sweet pepper: PS- PER1, PS96; anthurium: PS-AU5, PS-AU17; cat-tail willow: PS-CTW9, PS- CTW20, PS-CTW25; eustoma: PS-ES1, PS-ES8.	
race 2 strains [2]:	
Musa sp.: JS730, JS775, JS788, JS847, R633, R636.	
Other plant pathogenic bacteria [3]	
Erwinia species and strains	
E. carotovora subsp. carotovora Erc1; *E. chrysanthemi* S3-1; *E. cypripedii* EC155; *E. quercina* EQ101; *E. rhapontici* ER102; *E. rubrifaciens* ER103.	
Pseudomonas species and strains	
P. gladioli UCBPP550; *P. syringae* pv. apii PA102; *P. syringae* pv. tabaci PT124; *P. syringae* pv. erobotryae UCBPP258; *P. syringae* pv. coronafaciens UCBPP470; *P. syringae* pv. glycinea UCBPP527.	
Xanthomonas species and pathovars	
X. arboricola pv. celebensis XC145; *X. arboricola* pv. juglandis XJ123; *X. arboricola* pv. pruni XP10; *X. axonopodis* pv. citri XCI3-1, XW96; *X. axonopodis* pv. glycines XPS1, XPS3; *X. axonopodis* pv. phaseoli XP1, XP4; *X. campestris* pv. campestris XCC1-1, XCC33; *X. vesicatoria* XV2.	

1. The strains were isolated from different hosts, and obtained from Department of Plant Pathology, National Chung Hsing University, Asian Vegetable Research and Development Center, and Deptartment of Pesticide Application, Taiwan Agricultrual Chemicals and Toxic Substances Research Institute, Taiwan.
2. The DNAs of race 2 strains were obtained from Laboratoire de Phytopathologie, CIRAD-FLHOR, La Reunion, France, and Natural Resources Institute, University of Greenwich, Chatham, UK.
3. The strains were isolated in our laboratory or obtained from the International Collection of Phytopathogenic Bacteria, University of California.

*Hind*III-digested DNA was mixed with 1 μg of *Sal*I-digested *R. solanacearum* race 2 JS775 strain DNA. The mixture was denatured at 100℃ for 5 min and then allowed to reassociated for 18 h at 86℃ in 2.4 M phosphate buffer (pH 6.8). The reassociated DNA mixture was dialyzed extensively against 10 mM Tris-Cl-1mM EDTA (pH 8.0), precipitated with ethanol, and redissolved in 250 μl of sterile distilled water. Ligations were carried out overnight at 15℃, each reaction mixture containing 5 μl of subtracted mixture and 0.15 μg of phosphatase-treated *Sal*I-digested pBluescript SK(+) DNA (Stratagene Co., La Jolla, CA). Aliquots of the ligation mixture were transformed into competent *E. coli* DH5α cells, and transformants were selected on LB plates supplemented with ampicillin. Preparation of the insert DNA from the clones was carried out by PCR amplification using T7 and T3 oligonucleotide primers corresponding to sequences flanking the *Sal*I site of pBluescript SK.

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.* [9]. 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 PCR DIG probe synthesis kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Prehybridization, hybridization, and washing for Southern hybridization with DIG-labeled probes were performed at 68℃ according to the manufacturer's protocol. Subcloning into the sequencing vector pBluescriptSK(+) was carried out by routine techniques [9]. Double-stranded sequencing was performed by using an ABI Prism DNA Sequencing kit (Perkin-Elmer, Foster City, CA), based on the dideoxy chain termination method [10], and a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequence data were compiled and analyzed by using the computer programs of GCG (Genetics Computer Group, Madison, WI).

PCR amplification of *R. solanacearum*

Two oligonucleotide primers, IS*Rso19*-F (5'-TGGGAGAGGATGGCGGCTTT-3') and IS*Rso19*-R (5'-TGACCCGCCTTTCGGTGTTT-3'), were designed from nucleotide sequence of the 5'- and 3'-noncoding regions of IS*Rso19* for specific amplification of *R. solanacearum* race 2 strains. Specific PCR primers (PS-IS-F/PS-IS-R), based on 5'- and 3'-noncoding regions of IS*1405*, were used for detection of race 1 strains [6]. The IS*Rso19*-F/IS*Rso19*-R and PS-IS-F/PS-IS-R primer sets yielded 1,884 bp and 1,070 bp PCR products, respectively. Simple and multiplex PCR amplifications were performed with a GeneAmp®PCR system 2400 (Perkin-Elmer Corporation, Norway, CT, USA) in a 30-μl reaction mixture containing 2 mM $MgCl_2$, 0.5 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate and

1.25 U of *Taq* DNA polymerase (DyNAzyme II, Finnzymes Oy, Finland) by using the following program: 1 cycle of denaturation for 1 min at 94℃; 35 cycles consisting of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, and extension at 72℃ for 1.5 min; a final extension step at 72℃ for 2 min. Amplified DNAs were detected by electrophoresis in 0.8% (w/v) agarose gels and visualized by staining with ethidium bromide.

Nucleotide sequence accession number

The IS*Rso19* nucleotide sequence has been assigned GenBank accession number AF450275.

RESULTS AND DISCISSUION

Isolation of DNA fragments specific to *R. solanacearum* race 2

After subtractive hybridization, 21 colonies were obtained. To determine whether they were originated from and unique to *R. solanacearum* race 2, the insert DNAs were used as probes to hybridize *R. solanacearum* race 1 and 2, respectively. It was observed that a 1.0 kb *Sal*I insert DNA hybridized strongly to chromosomal DNA of *R. solanacearum* race 2 JS775 strain but not to race 1 strains. The same probe was then used to hybridize with *Bam*HI or *Eco*RI-digested total genomic DNA of other race 2 strains listed in Table 1. It was found that the probe hybridized to multiple fragments of all strains tested, indicating that 1.0 kb *Sal*I insert DNA was present in multiple copies in all tested *R. solanacearum* race 2 strains. Representative results are shown in Fig. 1.

1.0 kb *Sal*I insert DNA was then sequenced. Amino acid sequence deduced from the nucleotide sequence showed homology to transposases of IS*Rso6* form *R. solanacearum* race 1 strain, and IS*100* from *Yersinia pestis* (Table 2). For further characterization, the DNA fragment containing 1.0 kb *Sal*I fragment was isolated from *R. solanacearum* race 2 JS775 strain. The 4.0-kb *Bam*HI DNA fragments of race 2 JS775 strain corresponding to a hybridized fragment in Southern hybridization (Fig. 1) were eluted from agarose gel, ligated to *Bam*HI-digested pBluescript SK(+) vector and transformed into *E. coli* DH5α. The fragment containing 1.0 kb *Sal*I fragment was screened by hybridization, and then subcloned and sequenced. Sequence analysis revealed that the fragment contains a novel insertion sequence, which was designated IS*Rso19*.

Sequence analysis and feature of IS*Rso19*

The sequence of IS*Rso19* is shown in Fig. 2. Comparison of nucleotide sequence of IS*Rso19* and the deduced amino acid sequences of its gene products revealed that IS*Rso19* is homologous to the members of IS*21* family,

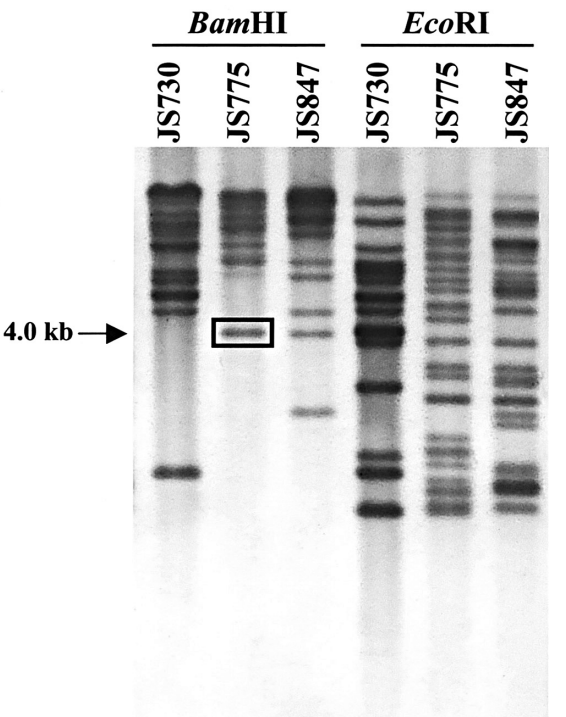

Fig. 1. Southern blot analysis of *Bam*HI or *Eco*RI-digested total genomic DNA from *R. solanacearum* race 2 strains, hybridized with a dig-labeled 1.0-kb *Sal*I DNA fragment. A 4.0-kb *Bam*HI fragment of JS775 strain eluted for cloning and further analysis is boxed. The size of band is indicated on the left.

but the extent of overall nucleotide sequence identity is only 39-58%. Thus, IS*Rso19* is a new member of IS*21* family. The identity scores of non-coding regions and amino acid sequence of open reading frames between IS*Rso19* and some members of IS*21* family are listed in Table 2.

IS*Rso19* is 1,956 bp in length and the overall G+C content is 64.37%. IS*Rso19* has 29-bp imperfect terminal inverted repeats (IRs) with 8-bp mismatches, and carry multiple repeated sequences at their ends that include part of the terminal IRs (Fig. 2). The IRs of IS*Rso19* terminate in the dinucleotide 5'-CA-3', which is one of features of IS*21* family [7].

Sequence analysis revealed that IS*Rso19* contains two open reading frames, *orfA* and *orfB*, which overlap for 4 bp. The *orfA* is followed by *orfB*, which is in phase-1 relative to *orfA*. The *orfA* has the capacity to encode a protein of 338 amino acids, if translation is initiated at ATG of position 81 and ended at position 1,097. Alternatively, the *orfA* may begin with a GTG at position 102, which is preceded at a distance of 5 bp by a sequence, AGGAGC, resembling the consensus Shine-Dalgarno ribosome-binding site sequence [12]. The *orfB* begins with a ATG at position 1,094 and extents to position 1,882, hypothetically encoding a gene product of 262 amino acids. The ORFA carries a motif related to the integrase DDE motif and a potential helix-turn-helix motif, while the ORFB carries two well conserved potential nucleoside triphosphate

Table 2. Comparison of nucleotide sequence of ISRso19 with members of IS21 family

IS name	5'-noncoding region		Coding region				3'-noncoding region		Accession number
			ORFA		ORFB				
	Length (bp)	Identity (%)	Length (aa)	Identity (%)	Length (aa)	Identity (%)	Length (bp)	Identity (%)	
IS*Rso6* of *Ralstonia solanacearum*	104	40.0	347	49.2	281	58.5	166	41.8	AL646058
IS*100* of *Escherichia coli*	85	43.7	340	44.6	252	42.4	82	50.0	AF218073
IS*100* of *Yersinia pestis*	86	41.2	340	39.6	252	53.9	86	52.7	Z32853
IS*21* of *Pseudomonas aeruginosa*	101	36.2	390	36.6	265	44.7	60	50.0	X14793
IS*Mav1* of *Mycobacterium avium*	194	41.2	493	33.5	264	26.5	67	46.2	AF125999
IS*640* of *Shigella sonnei*	113	36.2	315	34.9	-	-	36	41.6	X05956
IS*5376* of *Bacillus stearothermophilus*	92	46.2	400	33.9	251	42.6	60	40.0	X67861
IS*1631* of *Bradyrhizobium japonicum*	107	41.2	585	31.5	255	30.0	75	39.3	AB011021
IS*Bfl* of *Bacteroides fragilis*	190	43.7	582	24.2	263	31.5	88	34.7	U05888

binding domains. These domains are highly conserved within members of IS*21* family [7]. IS*21* transposition requires the istA and istB gene products [8]. By analogy, both ORFA and ORFB are, presumably, involved in IS*Rso19* transposition.

There are no consensus *E. coli* -10 and -35 promoter sequences in the region upstream of coding regions of IS*Rso19*. The actual promoter sequences have not yet been defined. As for IS*21*, the IS*21*-IS*21* junctions form a functional promoter that directs the transcription of the istAB operon. The upstream element provides the -35 region of the promoter, and the downstream element provides the -10 region [8]. An analogous phenomenon is possible for IS*Rso19*, since a potential -10 region (TGTAAA) is found at the left terminal inverted repeat and a potential -35 region (TTGACA) is present at the end of the element (Fig. 2). We could expect that an IS*Rso19* tandem with an intervening DNA sequence of about 10 bp would result in a functional promoter for the downstream *orfA* and *orfB*.

Specific primers for detection of *R. solanacearum* race 2 by PCR

Comparison of nucleotide sequence IS*Rso19* with that of insertion sequences of IS*21* family indicated that the identity of nucleotide sequence in 5'- and 3'-noncoding regions is only about 43% (Table 2). The diversity of 5' and 3'-noncoding regions makes IS*Rso19* easily distinguishable from other elements of IS*21* family. Thus, the 5'- and 3'-noncoding regions were used to design a primer set, IS*Rso19*-F/IS*Rso19*-R, specific to IS*Rso19* of *R. solanacearum* for PCR amplification. To determine the specificity of these primers, PCRs were carried out with DNAs of the strains listed in Table 1. The PCR amplification using IS*Rso19*-F/IS*Rso19*-R primers amplified a 1,884-bp DNA fragment from *R. solanacearum* race 2 but not from race 1 strains tested (Fig. 3A). No amplification was observed with other plant pathogenic bacteria tested, such as *Erwinia*, *Pseudomonas*, or *Xanthomonas* strains. Thus, IS*Rso19* isolated in this study is a novel insertion element that can be used as a specific marker for *R. solanacearum* race 2.

Multiplex PCR assay for differentiation of *R. solanacearum* race 1 and 2 strains

The 5'- and 3'-noncoding regions of IS*1405* have been used to design specific PCR primers (PS-IS-F/PS-IS-R) for detection of race 1 strains [6]. Thus, a multiplex PCR assay, which comprised specific primers targeted to IS*1405* and IS*Rso19*, respectively, was developed for rapid identification of *R. solanacearum* race 1 and 2 strains. The assay generated a unique 1,070-bp DNA fragment from each race 1 strain tested, and yielded a 1,884-bp DNA fragment from race 2 strains (Fig. 3B), proving again that each primer set was IS and race-specific. Since IS*1405* and IS*Rso19* are present as multiple copies in *R. solanacearum* race 1 and race 2, respectively, the multiplex PCR assay should enable more sensitive detection. Thus, these results suggest that this multiplex PCR is a rapid and sensitive method to detect and differentiate *R. solanacearum* race 1 and 2 strains.

Bacterial wilt of *Heliconia* can be caused by *R. solanacearum* race 1 and 2 strains [4,5]. This multiplex PCR will be very useful to identify the pathogens of bacterial wilt in *Heliconia* and other plants. In addition, the PCR primer sets will be further used to develop methods to detect the population of *R. solanacearum* in plant seeds and seedlings, irrigation water, or soil extracts.

Since other races of *R. solanacearum* have not yet been found in Taiwan, they were not tested in this study. It is unknown whether other races would also be amplified with the PCR primers. Furthermore, because the Moko disease has not been found in Taiwan, the DNAs of *R. solanacearum* race 2 strains used in this study were offered by foreign laboratories. Thus, the strain number of *R. solanacearum* race 2 used in this study may not be enough to draw definite conclusions about the specificity of the IS*Rso19*-F/IS*Rso19*-R primers to other *R. solanacearum* race 2 strains, but the

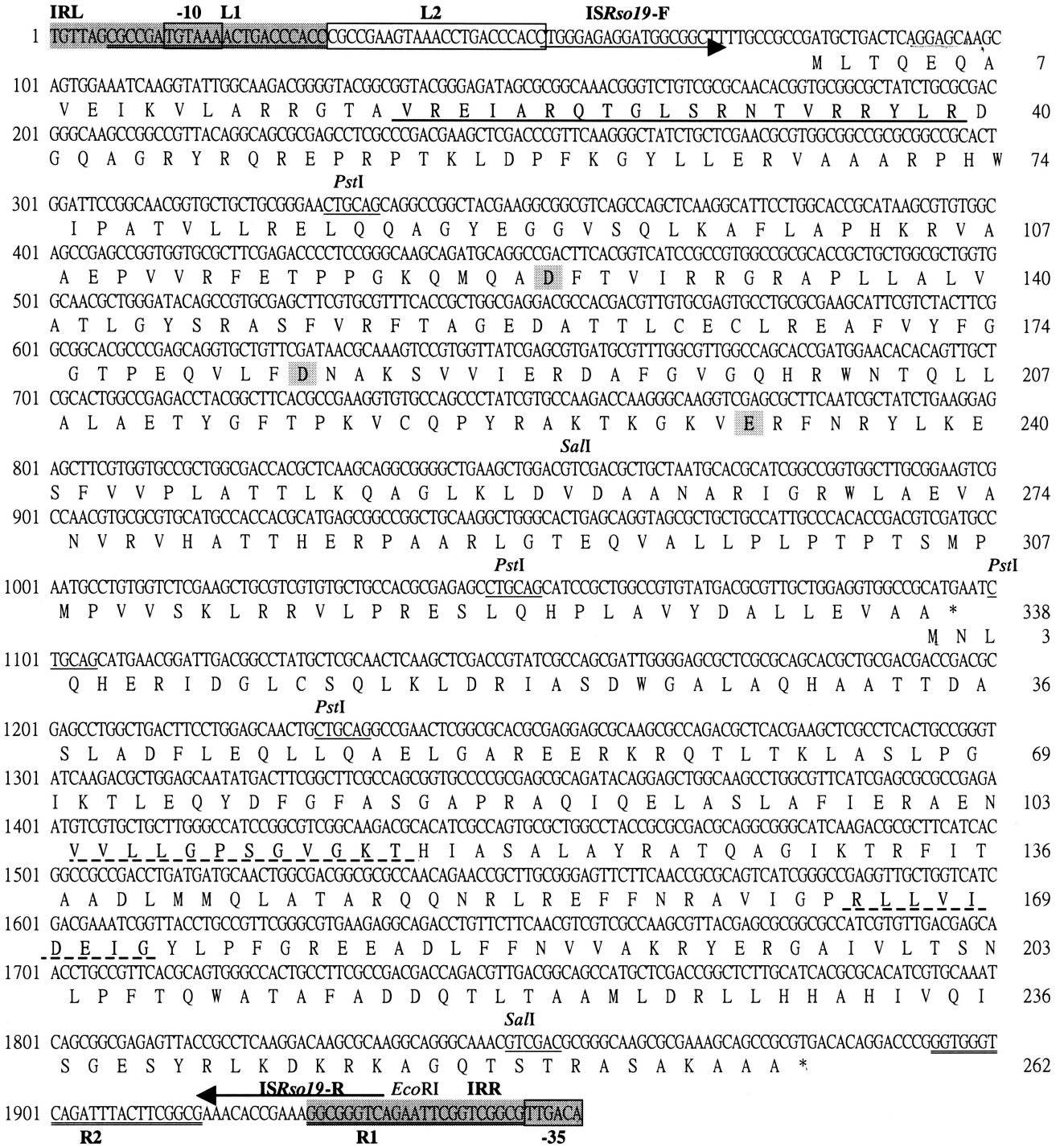

Fig. 2. Nucleotide sequence of IS*Rso19*. The deduced amino acid sequence of the ORF is given below the nucleotide sequence, and the termination codon is marked by an asterisk. The left and right terminal inverted repeat of IS*Rso19*, IRL and IRR are gray shaded. Multiple terminal repeated sequences, L2 is boxed whereas L1, R1 and R2 are double underlined. Putative promoter -10 and -35 hexamers are boxed. Helix-turn-helix domain and DDE motif of ORFA are underlined and shaded, respectively. NTP-binding site motif A and motif B of ORFB are also dotted underlined. The arrows denote the locations and orientations of the primers, IS*Rso19*-F and IS*Rso19*-R, designed to detect IS*Rso19* of *R. solanacearum* race 2 strains. IS*Rso19* is listed in the GenBank library under accession No. AF450275.

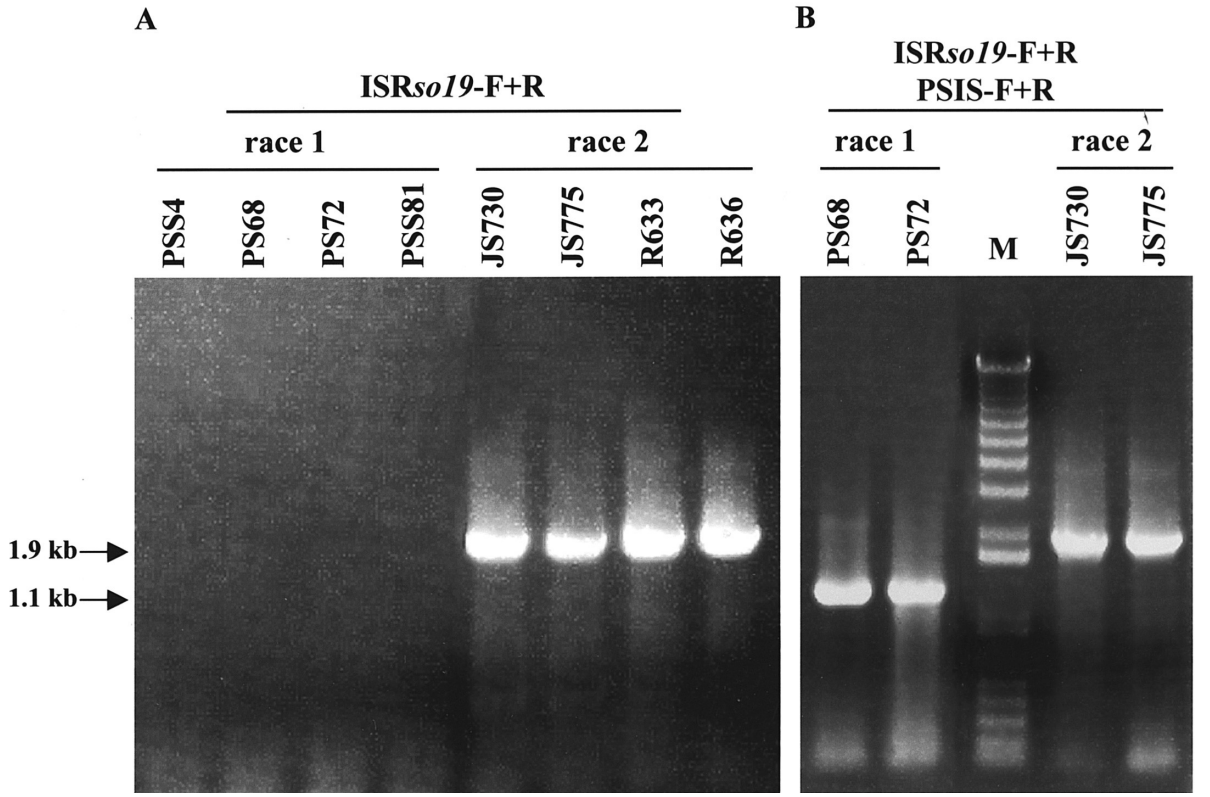

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) products from genomic DNAs of *R. solanacearum* race 1 or race 2 strains with (A) IS*Rso19*-F/ IS*Rso19*-R primer pairs or with (B) IS*Rso19*-F/ IS*Rso19*-R plus PSIS-F/ PSIS-R primer pairs, respectively. M, molecular size marker (1-kb DNA ladder, Gibco-BRL Life Technologies); sizes of PCR products are indicated to the left.

results reported here emphasized that insertion sequences can be used as a specific marker and applied to develop a PCR-based detection method for pathogenic bacteria. Because most plant pathogenic bacteria contain insertion sequences, it should be relatively easy to generate primer sets based on diversified 5'- and 3'-noncoding regions of insertion sequences.

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摘 要

李永安[1,2]、許眞妮[1]. 2003. 自青枯病菌分離出新插入序列IS*Rso19* 並應用於生理小種間的區分. 植病會刊12:57-64. ([1.] 輔仁大學生命科學系； [2.] 聯絡作者：電子郵件 bio1007@mails.fju.edu.tw，傳眞：+886-2-2902-1124)

自青枯病菌 race 2 中分離出一個新的插入序列-IS*Rso19*。經核酸序列分析結果，IS*Rso19* 與IS*21* 族群的成員較爲相似，但在 5'- 及 3'- 端的非轉譯部份與 IS*21* 族群的成員之間的相同度只有 43%。以 5'- 及 3'- 端的非轉譯部份的核甘酸序列設計聚合酵素連鎖反應 (PCR) 的引子組，可用以檢測青枯病菌 race 2。此引子組只對青枯病菌 race 2 擴增出預期的PCR 產物，而對青枯病菌 race 1 及其他植物病原菌均未擴增出任何 PCR 產物。因此 IS*Rso19* 爲青枯病菌 race 2 的專一性標記。另外，組合IS*Rso19* 及 IS*1405* 的引子組，可藉由multiplex PCR 的方式，區分青枯病菌 race 1 及 race 2。

關鍵詞：插入序列、IS*Rso19* 、IS*1405* 、青枯病菌