Acta Biochim Biophys Sin 2012, 44: 217–223 | © The Author 2012. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr129.
Advance Access Publication 29 January 2012

Original Article

Molecular detection of plant pathogenic bacteria using polymerase chain reaction single-strand conformation polymorphism

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The application of polymerase chain reaction (PCR) technology to molecular diagnostics holds great promise for the early identification of agriculturally important plant pathogens. *Ralstonia solanacearum*, *Xanthomoans axonopodis* pv. *vesicatoria*, and *Xanthomonas oryzae* pv. *oryzae* are phytopathogenic bacteria, which can infect vegetables, cause severe yield loss. PCR-single-strand conformation polymorphism (PCR-SSCP) is a simple and powerful technique for identifying sequence changes in amplified DNA. The technique of PCR-SSCP is being exploited so far, only to detect and diagnose human bacterial pathogens in addition to plant pathogenic fungi. Selective media and serology are the commonly used methods for the detection of plant pathogens in infected plant materials. In this study, we developed PCR-SSCP technique to identify phytopathogenic bacteria. The PCR product was denatured and separated on a non-denaturing polyacrylamide gel. SSCP banding patterns were detected by silver staining of nucleic acids. We tested over 56 isolates of *R. solanacearum*, 44 isolates of *X. axonopodis* pv. *vesicatoria*, and 20 isolates of *X. oryzae* pv. *oryzae*. With the use of universal primer 16S rRNA, we could discriminate such species at the genus and species levels. Species-specific patterns were obtained for bacteria *R. solanacearum*, *X. axonopodis* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae*. The potential use of PCR-SSCP technique for the detection and diagnosis of phytobacterial pathogens is discussed in the present paper.

Keywords PCR-SSCP; 16S rRNA; non-denaturing PAGE; Gram-negative bacteria

Received: September 14, 2011 Accepted: October 28, 2011

Introduction

Nucleic acid-based amplification systems like polymerase chain reaction (PCR) are promising methods for rapid identification of low numbers of plant pathogenic bacteria. The 16S rRNA sequence, which is highly conserved through the phylogenetic tree, is found in all prokaryotic organisms and is one of the most extensively studied target sequences [1]. The 16S rRNA gene also contains variable regions that have been used for the discrimination between the species and genera. *Ralstonia solanacearum*, *Xanthomonas axonopodis* pv. *vesicatoria*, and *Xanthomonas oryzae* pv. *oryzae* are economically important plant pathogenic bacteria distributed worldwide. Species and genus separation and identification are often necessary in the study of basic biology and epidemiology of diseases. *Xanthomonas axonopodis* pv. *vesicatoria* causes bacterial spot disease in tomato, *X. oryzae* pv. *oryzae* is the organism causing bacterial blight disease in rice, and *R. solanacearum* is responsible for bacterial wilt disease in tomato. The spread of these pathogens has the negative effect on yield.

Rapid and accurate microbial identification is an essential part in any pathogen inspection and survey program. Isolation of pathogens from diseased plants or seeds followed by morphological examination, biochemical/physiological characterization, hypersensitive response, and pathogenicity are the routine methods for detection and identification of plant pathogenic bacteria [2]. Classical methods for distinguishing these pathogens are based on morphological and growth characteristics [3] which are time consuming. An array of molecular approaches has been pursued in search for alternative techniques for more precise and rapid identification of the plant bacterial pathogens.

We made an attempt for directly identifying the bacteria based on the principle of single-strand conformation polymorphism (SSCP). SSCP is a technique designed to detect mutations in oncogenes and allelic variants in the human genome [4]. After PCR amplification of the target sequence, the amplified product is denatured into single-stranded DNAs (ssDNA) and subjected to non-denaturing polyacrylamide gel electrophoresis. Under non-denaturing

conditions, ssDNA has a secondary structure that could be determined by the nucleotide sequence. The mobility of the ssDNA depends on the secondary structure of the amplified product. The different positions of the bands of ssDNA on the gel indicate different sequences. PCR-SSCP is capable of detecting >90% of all single-base substitutions in 200 bp fragments [5]. Thus, the objective of this study was to rapidly identify plant pathogenic bacteria, and to generate the SSCP fingerprints of the individual genera of phytopathogenic bacteria via SSCP analysis.

Materials and Methods

Isolation of plant pathogenic bacteria including *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, and *X. oryzae* pv. *oryzae*

The phytopathogenic bacteria were isolated from infected plant material/seeds collected from farmers' field and local seed agencies, and were subjected to screening in the laboratory following different seed health testing methods like direct plating method and liquid assay method [6].

Direct plating method. Collected plant material/seed samples were surface-sterilized with 2% sodium hypochlorite followed by repeated washing with distilled water and blot dried, then plated directly on to the semi-selective medium like Tween B [7], yeast dextrose calcium carbonate (YDC) [8], and Kelman's triphenyl tetrazolium chloride (TZC) [9] media. Plates were incubated at $28 \pm 2^{\circ}C$ for 24–48 h. The yellow colonies with hydrolytic zones around the piece of plant material and seeds were observed for *X. axonopodis* pv. *vesicatoria*; yellow, circular, raised, mucoid colonies were observed for *X. oryzae* pv. *oryzae*; and typical mucoid, creamy white colonies with pink center were noted for *R. solanacearum*. After that these bacteria were isolated and subjected to biochemical/physiological, hypersensitive, and pathogenicity tests for confirmation of the pathogen.

Liquid assay method. The collected plant material/seeds were surface-sterilized as mentioned above. After that, they were macerated using sterile mortar and pestle in 10 ml of sterile distilled water. One milliliter of the supernatant was mixed with 9 ml of sterile distilled water to obtain a dilution of 10^{-1}, and the similar serial dilutions were done up to 10^{-5}. Fifty microliters of each dilution was plated on semi-selective media using Drigalski spreaders in triplicates. The plates were incubated at $28 \pm 2^{\circ}C$ for 24–48 h and observed for typical colonies of *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, and *X. oryzae* pv. *oryzae*.

Characterization of the pathogen

Characterization of the pathogens was done by subjecting the isolated bacterial colonies to various biochemical tests like Gram staining, KOH solubility test, starch hydrolysis, lipase activity, gelatin hydrolysis, and oxidative/fermentative metabolism of glucose. The strains were also subjected to hypersensitive test using tobacco (*Nicotiana tobaccum*), and pathogenecity test was carried out by using known susceptible host cultivars as per [10], with appropriate reference isolates.

PCR-SSCP studies

DNA extraction. Bacterial DNA was isolated using bacterial genomic DNA isolation kit from BangaloreGeNei (Bangalore, India) according to the manufacturer's instruction.

DNA amplification by PCR. The 16S rRNA was amplified by PCR for all the isolates using the primers: 16S forward primer (5′-TGGTAGTCCACGCCCTAAAC-3′) and 16S reverse primer (5′-CTGGAAAGTTCCGTGGATGT-3′), which were designed using Primer3 software. PCR was performed in a 0.2 ml PCR tube in volumes of 25 μl, containing 2.5 μl of 80–100 ng genomic DNA, 1 μl of 25 pmol of both the primers, 1 μl of 100 mM dNTP (25 mM each), 2.5 μl of PCR buffer, and 0.5 μl of 5 U Taq polymerase (BangaloreGeNei). The PCR was performed in an Eppendorf master gradient thermal cycler (Eppendorf, Hamburg, Germany). The procedure is as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 45 s at 72°C; the final extension at 72°C for 2 min, followed by cooling to 4°C until the sample was recovered. For all the PCR protocol, a reaction mixture containing a non-relevant bacterium—*Escherichia coli* DNA as well as sample containing without template DNA and non-infected host plant were used as negative control.

SSCP analysis. PCR products were first evaluated for purity and concentration by agarose gel electrophoresis. Five microliters of individual PCR product was mixed with 25 μl of denaturing buffer [95% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% bromophenol blue]. The mixtures were heated at 96°C for 10 min and then chilled with ice. Denatured PCR products were loaded on to 8% acrylamide–bisacrylamide non-denaturing gel with 8 ml of 40% acrylamide/bis, 4 ml of 10 × TBE (Tris borate 89 mM, boric acid 89 mM, 20 mM EDTA, and pH 8.0), 40 μl of tetramethylethylenediamine, 400 μl of 10% ammonium persulfate (APS), and 28 ml of water. APS were added as polymerization catalyser to 40 ml gel mix. The gel was cast using the gel sandwich set. Thirty microliters of each mixture was loaded, an aliquot of 25 ng

of ssDNA ladder were also loaded in both left and right lanes of the gel to facilitate comparison of SSCP patterns. Denatured PCR products were electrophoresed in prechilled 1 × TBE buffer at 200 V for 2 h at room temperature. After electrophoresis, polyacryalimde gels were peeled from the glass plates and silver staining was carried out [11]. The gels were first soaked in 50 ml of 10% ethanol for 10 min, and then placed with the same amount of 1% nitric acid for 3 min. After two brief washes with 100 ml distilled water, gels were stained in 50 ml of 2 p.p.m. silver nitrate (made from 100 × stock stored at 4°C) for 20 min followed by three times rinse with 200 ml of distilled water. Gels were developed by brief rinsing in 30 ml of 1 p.p.m. formaldehyde in 3% sodium carbonate until desired band intensity was reached. The stain was fixed in 1% acetic acid, once the SSCP patterns were visible. Images were captured for documentation and comparison between species. SSCP banding of individual isolates was done with the aid of ssDNA ladder.

Results

Isolation of plant pathogenic *X. axonopodis* pv. *vesicatoria, R. solanacearum*, *and X. oryzae* pv. *oryzae*

Out of the 66 tomato cultivars screened, 44 tomato cultivars showed the presence of *X. axonopodis* pv. *vesicatoria*, 56 cultivars had infected with *R. solanacearum* (**Table 1**) along with the suspected plant material. *Xanthomoans axonopodis* pv. *vesicatoria* colonies exhibited typical morphological characteristics such as yellow colonies with hydrolytic zones and *R. solanacearum* showed typical mucoid creamy white colonies with pink center. These pathogens were further purified by restreking them on their respective semi-selective media.

Out of 23 rice cultivars screened, 20 cultivars indicated the presence of *X. oryzae* pv. *oryzae* with the colonies showing yellow mucoid shining growth around the seed and plant material (**Table 2**). Both tomato and rice cultivars that were positive for *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, and *X. oryzae* pv. *oryzae* were further subjected to the liquid assay method and confirmed the presence of the three phytopathogenic bacteria.

Different isolates of *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, and *X. oryzae* pv. *oryzae* were further subjected to biochemical/physiological assays along with hypersensitive and pathogenicity tests to confirm the identity of *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, and *X. oryzae* pv. *oryzae*.

Biochemical characterization of bacterial isolates

The isolated bacterial strains showed Gram-negative characteristic by both Gram staining and also KOH solubility test (**Table 3**). The isolates *X. axonopodis* pv. *vesicatoria*

Table 1 Screening of different tomato cultivars for *X. axonopodis* pv. *vesicatoria* and *R. solanacearum* by the direct plating method

Sl No.	Cultivars	Results[a]	
		X. axonopodis pv. *vesicatoria*	*R. solanacearum*
1.	Sun Hybrid	+	+
2.	Arka-Abha	+	+
3.	Ashwini TA-4	+	+
4.	PKM-I	+	+
5.	Rohini-F1-TRA-A	+	+
6.	Hcl-IV	+	+
7.	TAG-XI0	–	+
8.	Sankranthi	–	+
9.	Rohini-TR	+	–
10.	Nimbusona	–	+
11.	POC-06	–	+
12.	CMM	+	+
13.	Local-I	+	+
14.	Local-II	–	+
15.	Local-III	+	+
16.	MPH-I	+	+
17.	TAC-F	+	+
18.	Ashwini-FI-TA-F	+	+
19.	Local-IV	–	+
20.	Local-V	+	+
21.	Abhinav	+	–
22.	Local-VI	–	–
23.	Local-VII	+	+
24.	Anoop	+	+
25.	Ashwini-FI-TA-B	+	+
26.	Abhinav	–	+
27.	Safal	–	+
28.	Indam	+	+
29.	Mrytunjaya	+	+
30.	Rasi	–	+
31.	Allrounder	–	+
32.	Naveen	–	+
33.	Vignesh	+	–
34.	Valley	+	–
35.	Sarpana	+	+
36.	PKM-1	–	–
37.	Arka Ashish	–	+
38.	Avinash	–	+
39.	Ashoka	+	+
40.	Nisco	+	+
41.	Vijaya	+	–
42.	Madanapalli	+	+
43.	Sasya	+	+
44.	Quality	+	+
45.	Sungro	+	+

Continued

Table 1. *Continued*

Sl No.	Cultivars	Results[a]	
		X. axonopodis pv. *vesicatoria*	*R. solanacearum*
46.	Golden	+	+
47.	Nandini	+	+
48.	Nautican	–	–
49.	ECL	+	+
50.	Ujwala	+	+
51.	Lakshmi	–	+
52.	Higeo	–	+
53.	Arunodaya	+	+
54.	OK seeds	+	+
55.	Indosem	+	+
56.	Leadbeter	+	+
57.	PHS	–	+
58.	Sree chakra 1	–	+
59.	Rasi	+	–
60.	Seedco	–	–
61.	Ashoka	+	+
62.	Malini	+	+
63.	Sree chakra 2	–	+
64.	Sulthan	+	+
65.	Solar	+	+
66.	Asha	+	+

[a]'+' and '−' indicate the presence and absence of phytopathogenic bacteria, respectively.

Sl No. indicates serial number.

and *R. solnacearum* did not show clear zone of hydrolysis, which indicated negative reaction for starch hydrolysis. Whereas for *X. oryzae* pv. *oryzae* clear zone of hydrolysis was observed indicating positive for starch hydrolysis (**Table 3**). The inoculated *X. axonopodis* pv. *vesicatoria* and *X. oryzae* pv. *oryzae* on Tween 80 agar plates showed the presence of white precipitate around the colonies of the bacteria, hence both the bacteria were positive for lipase activity. Whereas *R. solanacearum* did not show the presence of white precipitate around the colonies, hence the bacterium was negative for lipase activity (**Table 3**). After 7 days of incubation, *R. solanacearum* and *X. axonopodis* pv. *vesicatoria* did not liquefy gelatin media when compared with the control, hence they were negative for gelatin hydrolysis, whereas *X. oryzae* pv. *oryzae* showed the liquefaction of gelatin medium (**Table 3**). The isolated *R. solanacearum*, *X. axonopodis* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae* changed the color from green to yellow indicating positive for oxidation test, but in the fermentation test no color change from green to yellow was observed, thereby indicating a negative test (**Table 3**). Necrosis was evident in tobacco plants within 48 h of

Table 2 Screening of different rice cultivars for the presence of *X. oryzae* pv. *oryzae* by the direct plating method

Sl No.	Cultivar	*X. oryzae* pv. *oryzae*[a]
1	Gowrisanna	+
2	IR 64	+
3	1001	+
4	Thanu	+
5	Jaya	−
6	Ankursona	+
7	BR	+
8	Jyothi	+
9	Rasi	+
10	MR 1001	+
11	Sonamasuri	+
12	JGL	+
13	1010	−
14	Emergency sona	+
15	Doddi	+
16	BKB	+
17	Naga	+
18	Rajabhoga	+
19	64 sona	−
20	B.T. Mallige	−
21	Intana	+
22	Hemavathi	+
23	Tamilnadu sona	+

[a]'+' and '−' indicate the presence and absence of phytopathogenic bacteria, respectively.

Sl No. indicates serial number.

Table 3 Biochemical characterization of *X. axonopodis* pv. *vesicatoria*, *R. solanacearum*, and *X. oryzae* pv. *oryzae*

Biochemical tests[a]	Results		
	X. axonopodis pv. *vesicatoria*	*R. solanacearum*	*X. oryzae* pv. *oryzae*
Gram's reaction	−	−	−
KOH solubility	+	+	+
Starch hydrolysis	+	+	+
Lipase activity	−	−	+
Gelatin hydrolysis	−	w	+
O/F test	+/−	+/−	+/−
Hypersensitivity test	+	+	+
Pathogenicity test	+	+	+

[a]All the tests were conducted in four replicates and were repeated twice. + indicates positive reaction, − indicates negative reaction, and w indicates weak reaction.

infiltration with bacterial cells, whereas sterile distilled water infiltrated leaf regions did not show any change in leaf color. Tomato plants inoculated with *X. axonopodis* pv. *vesicatoria* and *R. solanacearum* showed bacterial spot and bacterial wilt symptoms, respectively. Rice seedling clipped with *X. oryzae* pv. *oryzae* showed typical symptoms of bacterial blight disease. Control plants inoculated with distilled water did not show any symptoms (**Table 3**).

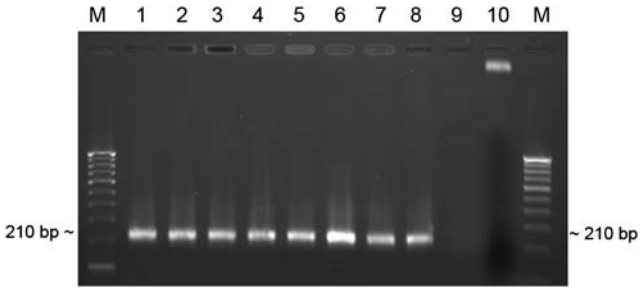

Figure 1 PCR products of different phytopathogenic bacteria with negative control Agarose gel electrophoresis of PCR products of *R. solanacearum* (lanes 1 and 2); *X. axonopodis* pv. *vesicatoria* (lanes 3 and 4); *X. oryzae* pv. *oryzae* (lanes 5 and 6) and *E. coli* (lanes 7 and 8), negative control (lane 9), and non-infected plant DNA (lane 10), 100 bp DNA marker (M).

Detection of *R. solanacearum*, *X. axonopodis* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae* using 16S rRNA pattern via PCR-SSCP

16S rRNA primers (FP 5′-TGGTAGTCCACGCCTAAAC-3′ and 5′-CTGGAAAGTTCCGTGGATGT-3′) were used to amplify the tested bacterial DNA in all the respective positive isolates to confirm the pathogens. The PCR product of 210 bp was obtained in all the isolates including the *E. coli* bacteria (**Fig. 1**), while the non-infected plant DNA and negative control did not show any amplification. The motilities of the ssDNA of all tested bacteria had species-specific pattern (**Figs. 2–5**). Electrophoresis of the

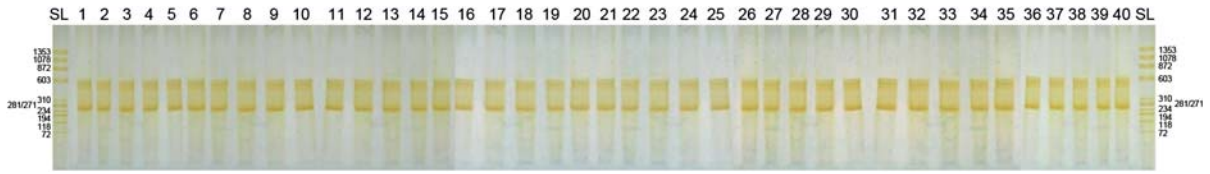

Figure 2 PCR-SSCP banding pattern of *R. solanacearum* isolates The DNA was isolated, PCR-SSCP was performed and banding patterns were visualized on non-denaturing polyacrylamide gels. Isolates 1–40 are different isolates of *R. solanacearum.* ssDNA marker (SL).

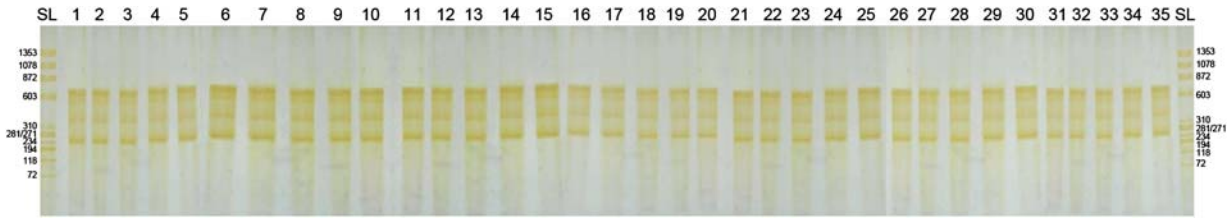

Figure 3 PCR-SSCP banding pattern of *X. axonopodis* pv. *vesicatoria* The DNA was isolated, PCR-SSCP was performed. and banding patterns were visualized on non-denaturing polyacrylamide gels. Isolates 1–35 are different isolates of *X. axonopodis* pv. *vesicatoria.* ssDNA marker (SL).

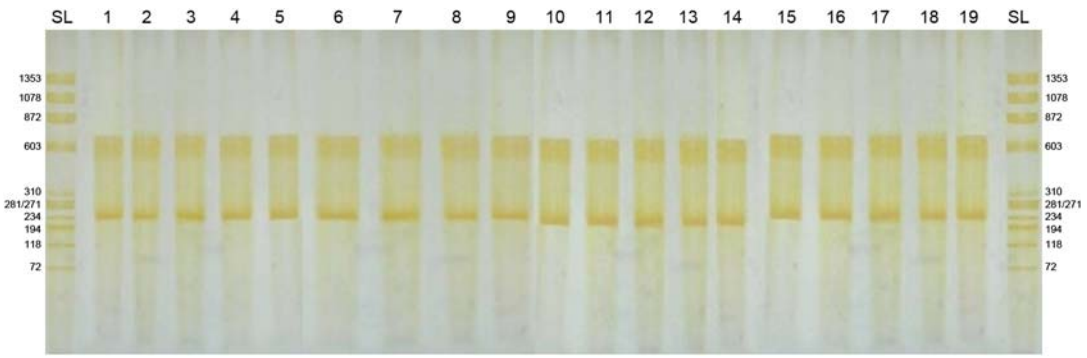

Figure 4 PCR-SSCP banding pattern of *X. oryzae* pv. *oryzae* isolates The DNA was isolated, PCR-SSCP was performed, and banding patterns were visualized on non-denaturing polyacrylamide gels. Isolates 1–19 are different isolates of *X. oryzae* pv. *oryzae.* ssDNA marker (SL).

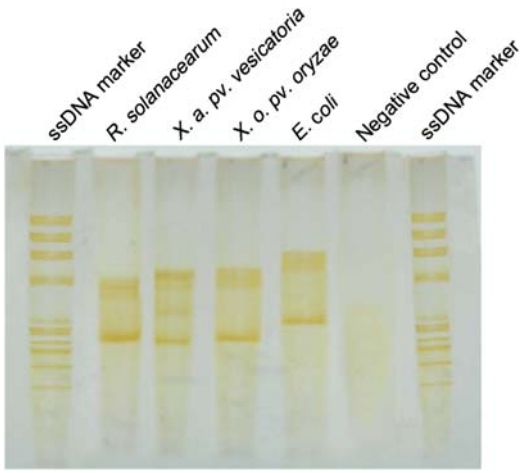

Figure 5 PCR-SSCP banding pattern of different phytopathogenic bacteria along with the non-relevant bacteria and negative control

denature 210 bp products resulted in two major bands for *R. solanacearum*, three major bands for *X. axonopodis* pv. *vesicatoria*, and two major bands for *X. oryzae* pv. *oryzae*, and the *E. coli* also showed the different banding pattern, which proves the sensitivity and the specificity of PCR-SSCP technique. The distance between the upper and lower bands varied among the species. These variations constituted simple and distinct SSCP patterns for individual species. These results indicated that the mobility of the molecules during SSCP analysis was distinct for each species of the genera of the bacteria tested.

Discussion

In the present study we isolated, identified, and characterized the bacterial spot disease causing pathogen *X. axonopodis* pv. *vesicatoria*, bacterial wilt causing pathogen *R. solanacearum*, and bacterial leaf blight causing pathogen *X. oryzae* pv. *oryzae*, which indicated the presence of these bacterial pathogens in many parts of tomato- and rice-growing regions of Karnataka, India. We carried out the direct plating method, by plating the infected plant materials and seed samples on their respective semi-selective media such as Tween B for *X. axonopodis* pv. *vesicatoria*, TZC for *R. solanacearum*, and YDC for *X. oryzae* pv. *oryzae*, resulting in the growth of yellow colonies with hydrolytic zones, typical mucoid creamy white colonies with pink center and yellow mucoid shining growth around the plant material, respectively. Colony morphology, biochemical/physiological tests, hypersensitivity test, and pathogenicity tests were included in the identification and confirmation of the isolated pathogen as *R. solanacearum*, *X. axonopodis* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae* [12,13].

Molecular diagnosis assumes an increasingly important role in the rapid detection and identification of plant pathogenic bacteria. The genetic variation of ribosomal genes in bacteria offers an alternative to traditional/conventional diagnostic methods. These genes, such as 16S rRNA, demonstrate conserved sequence regions ideal for primer targeting as well as regions variability useful for species identification [14]. In theory, since each bacterial species has a unique 16S rRNA sequence, all organisms can be differentiated from each other using PCR-SSCP technique. SSCP analysis of the 16S rRNA gene has been more recently used as an alternative to genomic sequencing for the identification of bacterial species [15–17]. SSCP analysis of 16S rRNA genes is a sensitive and specific method for species identification of phytopathogenic bacteria. We have developed PCR-SSCP fingerprints to detect the species-specific banding pattern for phytopathogenic bacteria in our laboratory. PCR-SSCP was performed according to the previous procedure [11]. The 16s rRNA of the three plant pathogens *R. solanacearum, X. axonopodis* pv. *vesicatoria*, and *X. oryzae* pv. *oryzae* exhibited different banding pattern due to the variations, which helped in discriminating these pathogens at the species level. To identify the variation and validation of PCR-SSCP technique in our study, we used *E. coli* DNA (which is a non-relevant bacteria) and without DNA sample as a negative control. To overcome lane-to-lane differences, PCR-SSCP resolution can be further improved by the addition of an internal ssDNA marker. This makes it feasible to precisely compare relative migration times. Although the DNA sequencing has become a common technique nowadays, it is still costly, especially when dealing with a large number of samples. Hence, we have shown that PCR-SSCP is a promising method for the identification of phytopathogenic bacteria. The use of PCR with universal primers and SSCP patterns can generally be applied as an identification method for a wide range of phytopathogenic bacteria. The only drawback of PCR-SSCP is that it detects the occurrence of base pair mutations in segments of DNA but does not give any information on the type of base changes, which has to be confirmed by sequencing. The results of the PCR-SSCP have to be further confirmed by sequencing of the genome.

In summary, this study provides essential data supporting that SSCP analysis is an effective molecular biological technique for distinguishing plant bacterial species, which could be extended to other similar plant pathogenic bacteria. This study also provides that 16S rRNA gene is an ideal DNA marker for species differentiation and identification of plant pathogenic bacteria.

Funding

This work was supported by the grants from University Grants Commission, Government of India under 11th Plan [36-281/2008 (SR)].

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