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Full Length Research Paper

Sensitive and specific detection of *Agrobacterium tumefaciens* in soil using a rapid polymerase chain reaction (PCR)

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One pair of primers was designed based on the sequence of *tmr* locus for specific and sensitive detection of *Agrobacterium tumefaciens*. Only the *A. tumefaciens* strain can produce the 236bp target fragment among the fourteen bacterial species that tested. The sensitivity of the specific PCR system was determined by a nested-PCR amplification which can numbered the copies of the template DNA. According to the results, it can give positive band when only 10^0 copies were in the template. The protocol was carried out for detection *A. tumefaciens* of twelve soil samples collected from six different gardens in Shanghai where crown gall happened. Two of the samples which collected from symptomless gardens also give the positive band. Based on the results we can make a conclusion that this pair of primers can be a useful tool in detecting *A. tumefaciens*, especially in detecting latent infection of this devastating pathogen.

Key words: *Agrobacterium tumefaciens,* detection, polymerase chain reaction (PCR).

INTRODUCTION

Agrobacterium tumefaciens, the soil born bacterium, is one of the most important species of *Agrobacterium* genus, can cause crown gall in most dicotyledonous and some monocotyledonous plants (Kerr and Panagopoulos, 1977). It cannot only infect the fruit trees such as peach and pear but also a big threat to the nursery industry, as infected plants often can not be sold.

Host range and oncogenic traits of *A. tumefaciens* are encoded by the Ti plasmid (pTi) a circular extrachromosomal DNA element. This plasmid contains 22 virulence genes (Vir region) that mediate the transfer of a portion of the Ti plasmid (T-DNA) into the plant cells. The T-DNA region is integrated into the plant genome. Subsequently, phytohormone genes (cytokinins and auxins) encoded on the T-DNA is over expressed in the plant cells (Zambryski and Schell, 1989). The overexpressed phytohormones induce uncontrolled plant cell proliferation and consequent formation of crown gall.

This pathogenic agrobacteria can symptomless survived in grape, rose and weeping fig (Tarbah and Goodman, 1987; Martì et al., 1999; Zoina et al., 2001). This is an important clue for the phytopathologist because the pathogen may be transmitted via vegetative propagation and even micropropagation system (Cooke et al., 1992; Poppenberger et al., 2002). Diagnosis is the first step to control a disease and up to now, detection of the pathogenic Agrobacteria strains is mostly done using traditional method that isolated the strains on selective medium and then tested the pathogenicity by inoculation into the herbaceous plants. This is time-consuming and is less appropriate for the diagnosis of latent infections. Moreover, the infection of plants by the crown gall causal agent, in contrast to other plant pathogens, can be caused by even a single bacterial cell (Lippincott and Heberlein 1965; Billing 1987). Therefore, an effective detection method for tumour-inducing agrobacteria in soil must be extremely sensitive.

And there are also several reports on detection of this

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Table 1. Bacterial strains used in this study

Bacterial strains	Species	Sources	Accession number	PCR detection
H16	*Agrobacterium tumefaciens*	ICMP	11272	+
Pb6	*Agrobacterium rhizogenes*	ICMP	11274	-
Vb8	*Agrobacterium vitis*	ICMP	11277	-
GMI1000	*Ralstonia solanacearum*	French Guyana	NC_003295	-
1JN2	*Bacillus subtilis*	Our lab	GU549436	-
2BGN8	*Serratia marcescens*	Our lab	HM161860	-
3YW8	*Myroides odoratimimus*	Our lab	GU549435	-
2JW6	*Stenotrophomonas maltophilia*	Our lab	GU549434	-
3YN16	*Enterobacter sp*	Our lab	GU549440	-
Z17	*Pantoea agglomerans*	Our lab	HM161866	-
3JW1	*Pseudomonas sp*	Our lab	GU991854	-
N2	*Burkholderia sp*	Our lab	HM161871	-
1JW4	*Acinetobacter sp.*	Our lab	GU991859	-
5JN2	*Ochrobactrum pseudogrignonense*	Our lab	GU991856	-
4GW19	*Pandoraea sp.*	Our lab	GU991852	-

kind of pathogen by serological and molecular techniques (Bishop et al., 1989; Burr et al., 1990; Cubero et al., 1999). Techniques based on bacteria DNA detection provides an opportunity for developing methods that are specific, sensitive, rapid and applicable for routine diagnosis of numerous soil samples. So far, a few PCR systems targeting the causal agent have been worked out. Especially using specific primers based on the nucleotide sequence of the Ti plasmid is one of the most powerful methods to detect tumorigenic bacteria in plant tissues and in the soil (Cubero et al., 1999). One of the first systems was based on amplification of the intercistronic region between *vir*B and *vir*G (Nesme et al. 1990). But these primers could only detect the strains with nopaline type pTi. Another two primers complementary to the *tmr* gene located within the T-DNA allowed for amplification of nopaline and octopine genes but not agropine pTi (Nesme et al. 1990). Dong et al. (1992) used two sets of primers which were also complementary to the *tmr* gene, but the sensitivity of detection was dependent on the tested strain and varied from 0.01 to 150 ng of target DNA. Also another problem is that PCR analysis is often interfered by the compounds released by plant, they can inhibit the DNA polymerase (John, 1992). So how to prevent the inhibition and provides high recovery of bacterial DNA is urgently needed.

Here, the aim of this study was to develop a specific, sensitive and rapid PCR-based method for detecting *A. tumefaciens* in soil. Such a method is necessary for early diagnosis for nursery production of fruit trees and other plant susceptible to crown gall.

MATERIALS AND METHODS

Bacterial strains and soil samples

The bacterial strains used in this study are listed in Table 1. All the strains were grown on LB medium at 28°C.

Soil samples were collected from 6 different gardens where the crown gall happened in different degree in Shanghai (E 121.445°, N 31.213°) at August 2009. Two of these gardens were grown pear and the other four were grown peach. All the soils were collected from the rhizosphere of the tree as described: first the soil around the stem was excavated by a scoop about 10 cm depths, and then the rhizosphere soil adhering on the root was collected use a small brush carefully. All the samples were put into small plastic bags and brought to the laboratory immediately for the further process.

Table 2. Two pair of primers designed in this study.

Primer name	Nucleotide sequence	Target (bp)
Tmr560F	TCGGGTCCAATGTTGTCCTC	560
Tmr560R	TCTGTTCTTGTCGGCGTGC	560
Tmr236F	TTATTGGAGTGCGGATTTTCGTT	236
Tmr236R	CGGATGTGATCTGGTTCTGGCTA	236

DNA extraction of the strains and soil samples

To extract genome DNA from the pure culture, bacterial cells were grown overnight at 28°C in LB broth. One milliliter of the culture was microcentrifuged, and total DNA was extracted by the Genome DNA Extraction Kit (Shanghai SBS Genetech Co. Ltd.). The results were proved by electrophoresis on 1% agarose gels.

The soil DNA was prepared following the instructions of the FastDNA Spin Kit for Soil (Mpbio industry, USA). Total DNA was electrophoresed on 1% agarose gels and approximately quantified according to the intensity of bands on the gel.

Primer design and specific PCR amplification

Primer tmr236 (Table 2) were designed according to the sequence of the tmr locus obtained from Genbank (GENE ID: 1224179). Primer tmr236F: 5'- TTA TTG GAG TGC GGA TTT TCG TT-3', primer tmr236R: 5'- CGG ATG TGA TCT GGT TCT GGC TA-3' (synthesized by Sangon bio-company Shanghai) 0.2 μmmol•L-

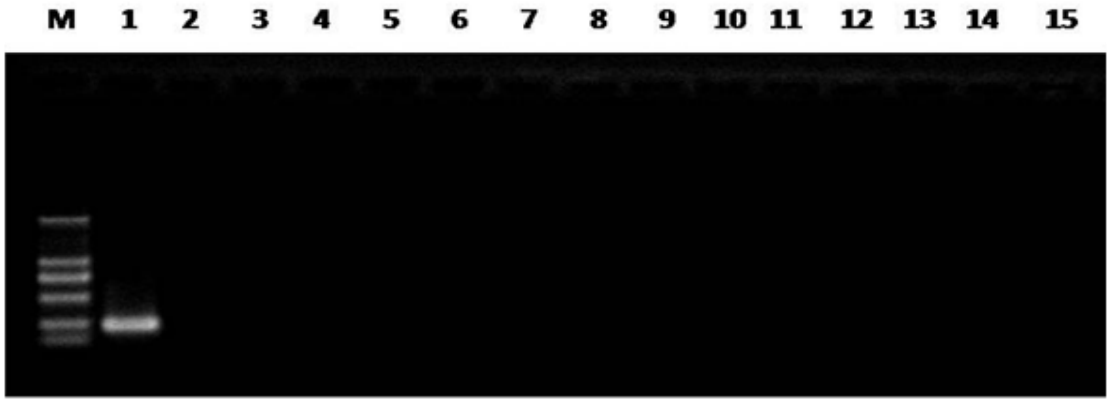

Figure 1. Specific PCR amplification of *A. tumefaciens* and strains of other species. M: 2000 bp DNA maker; 1: H16 (*A. tumefaciens)*; 2: Pb6 (*A. rhizogenes)*; 3: Vb8 (*A. vitis)*; 4: GMI1000 (*Ralstonia solanacearum*); 5: 1JN2 (Bacillus subtilis); 6: 2BGN8 (*Serratia marcescens*); 7: 3YW8 (*Myroides odoratimimus*); 8: 2JW6 (*Stenotrophomonas maltophilia*); 9: 3YN16 (*Enterobacter sp.*); 10: Z17 (*Pantoea agglomerans*); 11: 3JW1 (*Pseudomonas sp.*); 12: N2 (*Burkholderia sp.*); 13: 1JW4 (*Acinetobacter sp.)*; 14: 5JN2 (*Ochrobactrum pseudogrignonense*); 15: 4GW19 (*Pandoraea sp.*).

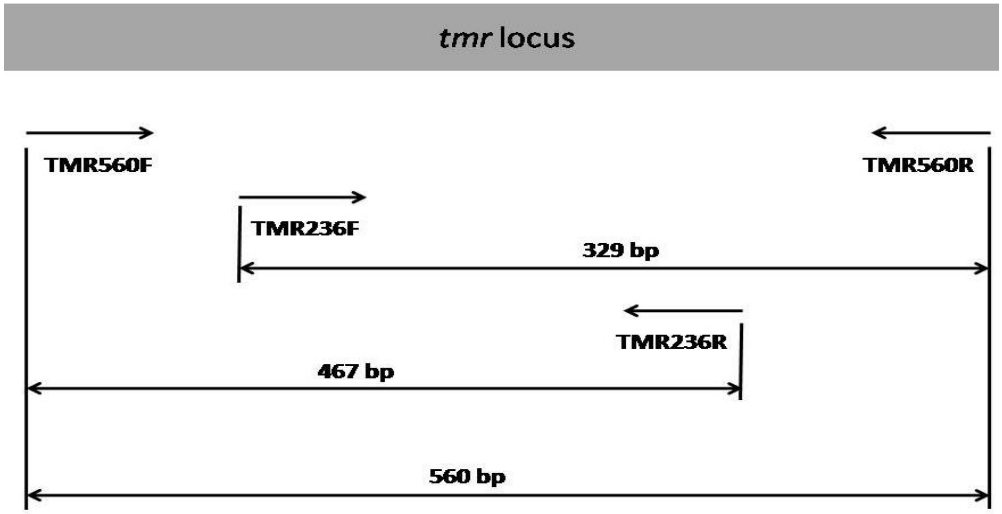

Figure 2. Positions and orientations of the primers used for detection of *A. tumefaciens* strains.

1; 1× PCR buffer; 0.2 mmol•L-1 dNTPs; 3.75 mmol•L-1 MgCl2; Tag polymerase enzyme 2.5 U and 1 μl template DNA was added in 25 μl system, respectively. PCR was done using BIO-RAD DNA Engine Peltier Thermal Cycle with an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C for 30 s, 72°C for 30 s, and final extension at 72°C for 10 min. The PCR products were detected by 1% agarose electrophoresis and strained by EB.

Sensitivity determination of the designed primers

First the specificity of the designed primers was evaluated by PCR amplification of eleven strains from other species (Table 1) that very common in the soil. Genome DNA extraction and PCR amplification was done as described before. And the results were detected by 1% agarose electrophoresis.

In order to evaluate the sensitivity of the designed primers, another pair of primer tmr560 was designed to amplify the region link to the target 236 bp fragment (Figure 2). The primer sequence are listed in Table 2 and the PCR amplification was done as described: Primer tmr560F and primer tmr560R (synthesized by Sangon bio-company Shanghai) 0.2 μmmol·L-1; 1 × PCR buffer; 0.2 mmol·L-1 dNTPs; 3.75 mmol·L-1 MgCl2; Tag polymerase enzyme 2.5 U and 1μl template DNA was added in 25 μl system respectively. PCR was done using BIO-RAD DNA Engine Peltier Thermal Cycle with an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 1 min, 56°C for 30 s, 72°C for 30 s, and final extension at 72°C for 10 min. The PCR product was linked to the pMD-19 plasmid (TAKARA BIO inc., Dalian) followed the instruction.

After multiplication in *Escherichia coli* top10 strain the total

Table 3. Soil samples collected from six different gardens in Shanghai.

Soil	Host	Symptom of crown gall	Target band	Soil	Host	Symptom of crown gall	Target band
1	Peach	+	+	7	Peach	-	-
2	Peach	-	-	8	Peach	-	+
3	Peach	-	-	9	Peach	-	-
4	Peach	-	-	10	Peach	+	+
5	Pear	+	+	11	Pear	-	+
6	Pear	+	+	12	Pear	-	+

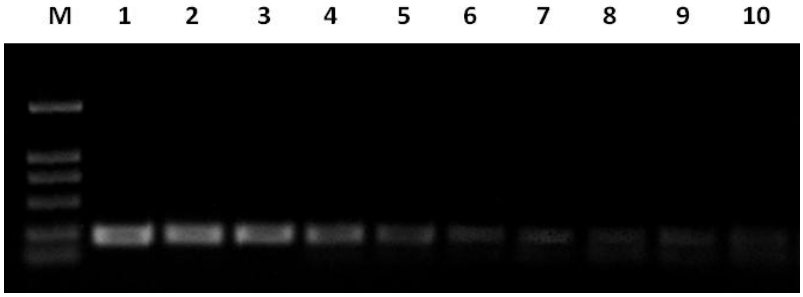

Figure 3. Sensitivity evaluation of the designed primers using a series dilution contains different copies of template DNA. M: 2000 bp DNA maker; 1-10: 10^9-10^0 copies of the 560 bp DNA template solutions.

plasmid was extracted by the AxtPrep™ Plasmid Miniprep Kit (Axygenbio Co., Ltd). The quality of the plasmid was measured by Nanodrop and the number of the 560 bp fragment copies was calculated according to the length of the inserted fragment and the whole vector. Based on the results, a series dilution that contains 10^0 to 10^9 copies of the fragment were prepared for the sensitive PCR amplification. PCR was done as described before and the gel was strained by EB and then pictured under 600 nm UV.

Specific detection of *A. tumefaciens* in soil

The designed primers were carried out to detect *A. tumefaciens* in the soil samples collected from six different gardens in Shanghai. The detail information of these soils was listed in Table 3, among them, some were infected by *A. tumefaciens* seriously and the others were symptomless infected. PCR was done as described before using the soil DNA for template. Products were electrophored by 1% agarose gel and pictured after EB straining.

RESULTS

Specificity of primers

On the basis of BLAST N analysis, the newly designed primers, tmr236F and tmr236R, did not show 100% homology to any known DNA sequence except the sequence of *tmr* locus of *Agrobacterium tumefaciens* Ti plasmid. As a result of PCR amplification, only the *A. tumefaciens* strain produced the 236 bp target fragment, none of the other fourteen species gave the positive band in comparison (Table 1, Figure 1). Even the two strains, Pb6 and Vb8 that from the same genus but different species also showed negative reaction.

PCR sensitivity

A series of dilution that contains the 560 bp DNA template from 10^9 to 10^0 copies were made to evaluate the sensitivity of the designed primers. All the diluted templates gave the target band compared to the blank control (Figure 3). According to the results we can make a conclusion that this primer is sensitive enough to produce the target fragment even from one copy of the template DNA.

Specific detection *Agrobacterium tumefaciens* in soil samples

Twelve soil samples collected from six different gardens in Shanghai were used to evaluate the sensitivity of the designed primers (Table 3, Figure 4). Among these soils, eight of them were collected from the rhizosphere of host plant that did not show any crown gall symptom. But three of them produced the target band which means it should be latent infected. And the four soils that collected from the infected tree also showed positive reaction.

According to the results we can say that this pair of

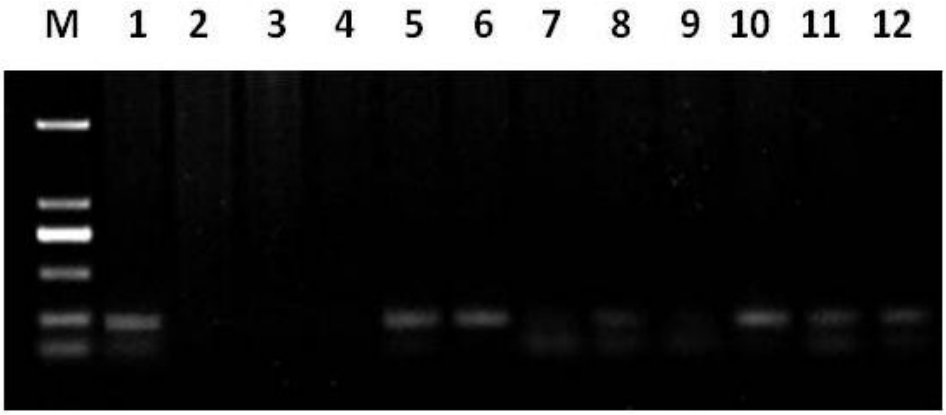

Figure 4. Sensitive and specific detection of *A. tumefaciens* in soil samples collected from Shanghai. M: 2000 bp DNA maker; 1-12: soil samples collected from different gardens in Shanghai, among them, 1, 5, 6 and 10 were collected from gardens where crown gall happened and the others were collected from symptomless gardens.

primer is sensitive enough to detect latent infection of *A. tumefaciens* in soil.

DISCUSSION

The crown gall caused by *A. tumefaciens* is becoming a big threat to nursery and fruit production. A sensitive and specific detection method is needed in early pathogen diagnosis for the symptomless host plant due to the specific infection mechanism. PCR methods have effectively been employed for sensitive and rapid detection and identification of phytopathogenic bacteria (Louws et al., 1999) because of its own advantages. PCR primers specific for and sensitive to phytopathogenic bacteria have been employed to study the efficiency of detection in infected plants or in the environment such as soil for early diagnosis of disease under natural conditions (Tsai and Olson, 1992; Louws et al., 1999).

Up to date, the specificity is still a difficulty in PCR detection. In this study, fourteen common bacterial species were used to evaluate the specificity of the designed primers. As expected, none of them could produce the positive band compared to the *A. tumefaciens.* These eleven species include the commonly soil-born disease *Ralstonia solanacearum*, and *Bacillus sp, Pseudomonas sp, Enterobacter sp*, which is regarded as the most dominant species in soil environment. Even the *Agrobacterium rhizogenes* and *Agrobacterium vitis* species also could not amplify the positive band. These two species are closely relative to *A. tumefaciens* according to their phytopathogenic characteristics and could caused crown gall on other plants (Kerr and Panagopoulos, 1977). As reported, attempts also have done to distinguish the different species which belong to *Agrobacterium* use PCR method (Pulawska et al., 2006; Bini et al., 2008). As shown in the results, this pair of primer gives us an unexpected finding that it can distinguish *A. tumefaciens* from the other closely species *A. rhizogenes* and *A. vitis.*

Our procedure showed very high sensitivity in the artificial system since it can give positive band from only one copy of the template DNA. This is a big progress compared to the reported detection system which could only detected from 10^{2-3} CFU/g soil or plant tissue (Lim et al., 2009; Picard et al., 1992; Sachadyn and Kur, 1997). Latent diagnosis is more important since the symptomless seeding and soil are the main sources of this disease. And according to the specific invasion mechanism it is hard to control in case the symptom emerged.

Based on the high sensitivity and specificity, our procedure was carried out to detected *A. tumefaciens* strains in six different gardens in Shanghai. As expected, not only the soil samples collected from symptom gardens but also the symptomless ones could produce the positive band. This result gives us important information that control measures should be carried out immediately in order to prevent uncertain loss.

Additionally, our procedure can be completed within 6 hours accompany with fast DNA extraction kit. It is very convenient and easy to be developed in actual diagnosis.

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