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Detection Method for Potato Brown Rot and Ring Rot by PCR

Ri Won Jin

Abstract The specific primers for detection of pathogenic bacteria causing potato disease such as Brown rot and Ring rot with the amplification of ribosomal sequences by PCR were designed and synthesized and optimum PCR detection condition was elucidated. The method of nucleic acid extraction for detection of diseases by bacteria in potato was selected and the detection limitation of bacterial DNA was elucidated. This result can be applied to the production of non-disease potato.
Key words potato, bacterial diseases, PCR, primer

Introduction

Potato is subjected to diseases of various kinds.

In potato, several bacterial diseases cause serious yield losses and grow obstacles. Brown rot is caused by the *Ralstonia solanacearum* and ring rot is caused by the *Clavibacter michiganensis* subsp. *sepedoncus* [2, 3, 6].

As these diseases are the main limiting factor, the production decrease rate by it is high as 50% [1].

At present, no effective agrochemicals exist for control the potato bacterial diseases in the worldwide [9].

So, the early detection by means of sensitive diagnostic methods is the main way to control them [4].

There are several detection methods such as symptom observation, nucleic acid amplification and immunologic assay. PCR is one of reasonable method [5].

Therefore, we researched the detection method for rapid and exact diagnosis of bacterial disease in potato by using PCR.

1. Materials and Method

1.1. Materials

Potato tubers showing clear symptoms and pathogenic bacteria strains causing brown rot and ring rot which are isolated, identified and stored before, were used in experiment.

1.2. Reagents and experimental instruments

The $MgSO_4 \cdot 7H_2O$, K_2HPO_4, Tris-HCl, DNeasy Plant Mini Qiagen Kit, Tris-HCl, BSA, EDTA, SDS, CTAB, NaCl and etc. were used as reagents.

PCR Engine, thermostat, water bath, incubator, vortex mixer, micro-centrifuge, gel dock, electrophoresis apparatus, micro-pipette and etc. were used as instruments.

1.3. Methods

Nucleotide sequences of relative bacteria were collected from NCBI data base. By using design software "Primer Designer 4(version 4.20)", the specific primer for detection of pathogenic bacteria causing potato diseases with the amplification by PCR were designed and synthesized from 16S rRNA sequences for brown rot, and from 16S-23S rRNA & 23S rRNA sequences for ring rot, respectively [7, 8, 10].

PCR conditions with these primers were established using the standard procedure [11, 12].

2. Results and Discussion

First of all, we have designed the specific primers to potato bacterial wilt.

By surveying the complete sequence of pathogenic bacteria *Ralstonia solanacearum*, causal agent of potato bacterial wilt, we selected species-specific sequences from brown rot 16S ribosomal RNA genes, and designed the primer pairs from these sequences.

When we performed BLAST explorer on designed primer pairs using software DNA Star, satisfactory results were obtained. As a result, the designed brown rot specific primer pairs for PCR detection and the characteristics are shown in table 1. When using designed primer pairs, the products size of PCR is 286bp.

Table 1. Designed primer pairs

Name	Primer sequence(5′→3′)	Tm/℃	Size/bp
PRal-F	GGGGGTAGCTTGCTACCTGCC (21)	60.0	286
PRal-R	CCCACTGCTGCCTCCCGTAGGAGT (24)	62.2	

Using same procedures, we designed the primer pairs for detection of ring rot.

In the case of ring rot, we selected the 16S ribosomal genes and 23S ribosomal genes sequences as design regions. Design methods are same as in the case of brown rot. In the case of ring rot, PCR product was 214bp(table 2).

Table 2. Designed primer pairs

Name	Primer sequence(5′→3′)	Tm/℃	Size/bp
PCla-F	CCTTGTGGGGTGGGAAAA (18)	61.0	214
PCla-R	TGTGATCCACCGGGTAAA (18)	59.2	

Next, with each designed primer pairs of brown rot and ring rot we have studied to elucidate the suitable PCR conditions.

As an experimental result, the suitable annealing temperature and time in brown rot were 62℃ and 45s.

When amplified 30 cycles, the best products were obtained by adding the 250nmol/L of each primer and 200μmol/L dNTPs to PCR solutions respectively.

In the case of ring rot, suitable annealing condition was to perform 30 cycles of PCR, keeping the temperature of 62℃ for 60s. The concentrations of primers and dNTPs were same as in the brown rot.

The composition of PCR solution which we have studied was shown in table 3.

Table 3. PCR solution composition

Potato brown rot			Potato ring rot		
Component	Quantity	Concentration	Component	Quantity	Concentration
10×Buffer	2.0μL	1×	10×Buffer	2.0μL	1×
25mmol/L $MgCl_2$	1.6μL	2.0mmol/L	25mmol/L Mgcl2	1.6μL	2.0mmol/L
10mmol/L dNTPs	1.6μL	200μmol/L	10mmol/L dNTPs	1.6μL	200μmol/L
Primer 1(10μmol/L)	0.5μL	250nmol/L	Primer 1(10μmol/L)	0.5μL	250nmol/L
Primer 2(10μmol/L)	0.5μL		Primer 2(10μmol/L)	0.5μL	
TaqPolymerase (5U/μL)	0.1μL	1.25U/μL	TaqPolymerase (5U/μL)	0.1μL	1.25U/μL
Sample DNA (50ng/μL)	2.0μL	5ng/μL	Sample DNA	2.0μL	5ng/μL
BSA(0.1%)	0.4μL	0.002%	BSA(50ng/μL)	0.4μL	0.002%
ddH_2O			ddH_2O		
Total PCR 20μL			Total PCR 20μL		

Established programs of PCR are as follows (table 4).

Table 4. Programs of PCR

Brown rot			Ring rot		
Preheating	95℃, 5min		Preheating	94℃, 5min	
Transforming	95℃, 30s	30 cycle	Transforming	94℃, 30s	30 cycle
Annealing	62℃, 45s		Annealing	62℃, 60s	
Reaction	72℃, 1min		Reaction	72℃, 3min	
	72℃, 10min			72℃, 10min	
Storage	4℃		Storage	4℃	
Electrophoresis			Electrophoresis		

And we selected the optimal extraction method of nucleic acids.

Among 4 methods, 3 methods except heat treatment gave good results (table 5).

An addition[11] 0.1% bovine serum albumin (BSA) to reaction solution give positive influence to PCR and it is possible to increase more the detection sensitivity.

Table 5. Purity of nucleic acids isolated with several methods

Method	Tuber		Bacterial culture	
	Brown rot	Ring rot	Brown rot	Ring rot
Kits	1.975 68	1.827 55	1.856 62	1.815 24
Heat treatment	1.684 94	1.514 92	1.647 53	1.744 73
SDS	1.870 50	1.946 52	1.907 41	1.800 75
CTAB	1.910 37	1.892 19	1.850 48	1.901 88

And we recognized that PCR has high detection sensitivities on bacterial in samples. Performance of post-enrichment culture of bacterial before PCR is an important way to increase the detection sensitivity.

Through post-enrichment procedure, we can detect brown rot and ring rot at concentrations of 10^3CFU/mL respectively (Fig. 1 and 2).

Through above research, we have established the PCR detection methods on brown rot and ring rot of potato.

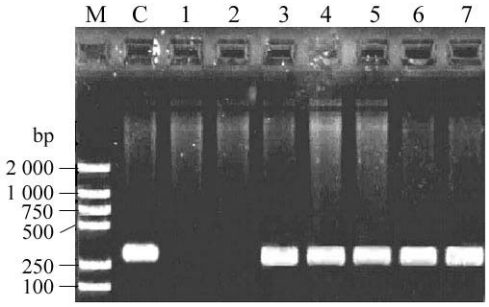

Fig.1. PCR detection sensitivity at potato brown rot concentrations
M: maker(Ladder), C: positive control,
1－7: concentration of bacteria(10^1～10^7CFU/mL)

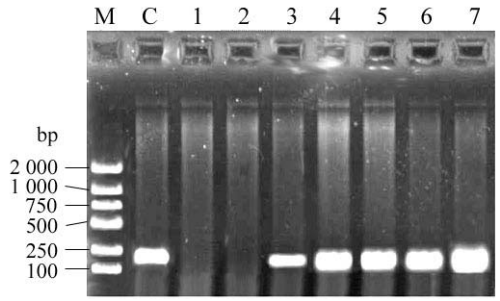

Fig 2. PCR detection sensitivity at potato ring rot concentrations
M: maker(Ladder), C: positive control,
1－7: concentration of bacteria(10^1～10^7CFU/mL)

Conclusion

The specific primers for testing brown rot and ring rot in potato were designed.

The amplified product size of primer pairs which were designed on the basis of 16S rRNA sequences of potato brown rot was 286bp. In case of ring rot, the size of amplified product was 214bp.

PCR detection conditions of brown rot and ring rot were elucidated.

We have selected rational methods of nucleic acid extraction for the PCR detection of potato brown rot and ring rot and elucidated the detection limitation of bacterial DNA in PCR.

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