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A Simple Detection of Sweetpotato Feathery Mottle Virus by Reverse Transcription Polymerase Chain Reaction

Jae-Hun Jeong[1,2*], Debasis Chakrabarty[2], Young-Seon Kim[3], Jong-Seon Eun[4], Yong-Eui Choi[1], Kee-Yoeup Paek[2]

[1]Korea Ginseng Institute, Chung-ang University, Anseong-shi 456-756, Korea; [2]Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, Korea; [3]Department of Horticultural Industry, Na Provincial College of Jeomnam, Changheung 529-850, Korea; [4]Department of Horticulture, Research Institute of Bioindustry, Chonbuk National University, Jeonju 561-756, Korea

Abstract

A reverse transcription polymerase chain reaction (RT-PCR) protocol was developed using two specific 22-mer primers located in coat protein gene of SPFMV. A 411 bp PCR-product was detected in virus infected plants as well as tissue culture raised sweet potato but not in healthy plants. For optimization of RT-PCR protocol, the optimum crude nucleic acid concentration, annealing temperature, primer concentration and numbers of PCR-cycle for maximum sensitivity and specificity were determined. The optimum condition for RT-PCR was as follows: RT-PCR reaction mixture was one-step mixture, containing 50 pmol of primer, 30 units of reverse transcriptase, 5 units of RNasin, and the crude nucleic acid extracts (200 ng). In RT-PCR, cDNA was synthesized at 42°C for 45 min before a quick incubation on ice after pre-denaturation at 95°C for 5 min. The PCR reaction was carried out for 40 cycles at 96°C for 30 sec, 63°C for 30 sec, 72°C for 1 min, and finally at 72°C for 10 min. The viral origin of the amplified product was confirmed by sequenceing, with the sequence obtained having 95-98% homology with published sequence data for SPFMV. The benefits of this RT-PCR based detection of SPFMV would be simple, rapid and specific.

Key words: Reverse transcription polymerase chain reaction (RT-PCR), *Sweet Potato Feathery Mottle Virus* (SPFMV), specific primer, sequencing, virus detection

* Corresponding author, E-mail: biojeong@hotmail.com
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Introduction

Sweet Potato Feathery Mottle Virus (SPFMV) is a member of the family Potyviridae. These are plant viruses with a single (+) sense RNA genome of about 11.6 kb in length which has a poly A tail at its 3' termini and a 5' linked protein (VPg) (Moyer and Cali 1985). Many surveys have shown that the SPFMV is the most prevalent and widespread pathogen in the world where sweet potatoes are cultivated (Cali and Moyer 1981; Moyer and Salazar 1989; Colinet and Kummert 1993). SPFMV generally induces irregular chlorotic patterns, so called feathering associated with faint or distinct ring spots that have purple-pigmented borders (Moyer and Salazar 1989).

Detection of virus in plants is usually based on the use of antisera and/or monoclonal antibodies in an enzyme-linked immunosorbent assey (ELISA), but this method suffers from a lack of sensitivity (Koenig and Paul 1982; Yoon et al. 1991). Nucleic acid amplification techniques can increase the sensitivity of the detection of plant viruses, compared with serological methods. The use of RT-PCR as a sensitive method for the detection of RNA viruses has been reported (Griesbach 1995; Hu et al. 1995; Li et al. 1998; Singh and Singh 1998; Jeong et al. 2001). In this study, it was shown that the feasibility of simple detection of SPFMV in sweet potato plants.

A 411bp sequence in the read through region from the coat protein gene (Figure 1) of SPFMV was chosen for amplification. The nucleic acid sequence of SPFMV published by Ryu et al. (1998) was used as the basis for the design of two primers for use in reverse transcription and PCR amplification. During the course of this study, some factors affecting RT-PCR (crude nucleic acid concentration, annealing temperature, primer concentration and PCR cycle) were experienced and

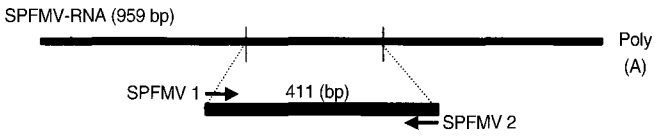

Figure 1. SPFMV-RNA for coat protein, showing the location of specific primers (SPFMV 1, 2) used in this study.

optimum conditions for maximum sensitivity and specificity for PCR amplification are described.

Materials and Methods

Sources of viruses and plants

The SPFMV isolated originally from an infected sweet potato plant (Ipomoea batatas cv. Kwandong 95) was used as source of viruses (Kim et al. 1998). Sources of plants were tissue culture regenerated sweet potato, virus infected plants (used as positive control), and collected from commercial sweet potato farms of Chonbuk province in Korea. Healthy plant was used as a negative control.

Total nucleic acid extraction

Tissue samples for PCR were prepared from 20 mg of fresh leaf tissue homogenized in a 1.5 mL micro centrifuge tube with sterilized drill tip containing 200 μL of extraction buffer (3 M guanidium thiocyanate, 10 mM 2-ethane sulfonic acid pH 6.5 and 30 mM EDTA) (Lee et al. 1996). The mixture was extracted once with 200 μL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) solution and centrifuged at 12,000 rpm for 10 min. The 100 μL of upper aqueous phase was transferred to a new tube. The aqueous phase was then mixed with 60 μL of isopropanol, centrifuged at 12,000 rpm for 15 min at 4°C. The resultant pellet of nucleic acid was washed in 70% ethanol and then dried. The pallet was again resuspended in 200 μL of diethylpyrocarbonate-treated water at 70°C until use.

Oligonucleotide primers

Two specific 22 bp primers were designed based upon the nucleotide sequence of SPFMV coat protein (EMBL accession AF439637, AJ539130, AJ310201, AJ310202, AJ010707, AJ010703, AJ010698, AF015540, S43450, D38543, D86371, AJ515379, AJ515378, AJ539132, AJ539131, Z85996, AJ010706, AJ010704, AJ010702, AJ010701, AJ010700 and Ryu et al. 1998). The upstream primer SPFMV1 (5'-ATA GTG GGG GCA TCA TCA AAG G-3') was corresponded to nucleotides 202 to 223 of the SPFMV RNA. The downstream primer SPFMV2 (5'-CCT AAA AGT AGG CAC TGC ATG G-3') was complementary to bases 591 to 612 of the viral RNA. The set of primers was used to amplify a 411 bp DNA fragment for detection of SPFMV from sweet potato plants.

cDNA synthesis and PCR amplification

Reverse transcription-PCR was carried out at 42°C for 45 min in 10 mM Tris-HCl buffer (pH 8.3) containing 4 μL sample RNA, 20 pmol primer, 2.5 mol dNTP, 30 units reverse transcriptase (Bioneer, Korea), 5 units RNasin, and 1 unit *Taq* DNA polymerase (Bioneer, Korea). The reaction was predenatured for 2 min at 96°C, denatured for 30 sec at 96°C, annealed for 30 sec at 63°C, and then extended for 1 min at 72°C for a total of 40 cycles in the DNA thermal cycler (Perkin Elmer Model 480, USA). For optimization of PCR program, different concentration of nucleic acid (100-300 ng), annealing temperatures (55, 60 and 63°C), primer concentration (20-100 pmol) and PCR cycles (35, 40 and 45) were tested for maximal sensitivity and specificity.

Gel electrophoresis of PCR products

PCR products were analyzed by electrophoresis through 1.7% agarose gels in TBE buffer (89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA pH 8.0). An aliquot of 10 μL from a 20 μL PCR reaction volume was loaded onto each gel. The gels were run in a mupid gel tank (Mupid-2 Cosmo Bio, Japan) at 50 V for 40 min. Gels were stained after electrophoresis with ethidium bromide and examined on a UV transilluminator.

PCR product analysis by sequencing

The 411 bp fragment from RT-PCR of nucleic acid from infected plants was eluted with gene clean kit (Bio 101, USA). And this 411 bp fragment was used directly as template DNA for direct DNA sequencing (ABI Prism 377 sequencer, Perkin Elmer, USA) using the same primers. Homology of the sequences was compared with published sequence data for SPFMV (Gene Bank).

Results and Discussion

Using crude nucleic acid from infected sweet potato plant and for RT-PCR, products of the expected size of 411 bp was observed in agarose gels for SPFMV. Non-specific bands were rarely visible using the conditions described. Furthermore, no amplification products were seen when crude nucleic acid from healthy plants were used as the template in the presence

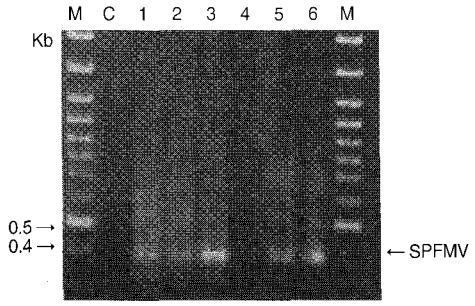

Figure 2. Detection of SPFMV by one step RT-PCR. Ethidium bromide-stained agarose gel of RT-PCR products using various templates are shown. The arrow indicates the position of the 411 bp PCR product. Lanes M; 100 bp DNA ladders, Lane C; nucleic acid extracted from healthy sweet potato plant, Lanes 1-3; nucleic acid extracted from SPFMV infected sweet potato plants, lanes 4-6; nucleic acid extracted from randomly selected tissue culture raised sweet potato plants for diagnosis of SPFMV (note lane 4, no infection was detected).

SPFMV primers (Figure 2). Preliminary results using the conditions for RT-PCR by Jeong et al. (2001) highlighted some problems and gave faint band covered with smearing after RT-PCR (Figure 2). Factors that influence the amplification of target loci have been investigated with specific reference to amplification of coat protein gene of SPFMV. The major factors involved in product amplification are discussed below. Comparison of crude nucleic acid concentration and their amplification by RT-PCR suggested that 200 ng nucleic acid extracts improved the yield of amplification product but gave 411 bp band covered with thick smearing due to non-specific products (Figure 3A). This result suggested that the dense zone of non-specific products between 200-800 bp might be due to uncontrolled extension of specifically annealed primers. This prompted us to study different annealing temperatures during PCR reaction to improve the protocol. The yield of amplification product increased when 63°C was used, but the reaction failed to produce visible amplicon compared to 55°C annealing temperature. Annealing temperature of 60°C yielded lighter product bands covered with smearing (Figure 3B). Figure 4A shows the amplification products of the 411 bp target loci of SPFMV using different primer concentrations (in all reactions crude nucleic acid concentration and annealing temperature was kept constant: 200 ng and 63°C respectively). All primer concentrations showed the amplification of the target loci but with different degrees of amplification except for 100 pmole where no PCR reaction was observed (Figure 4A). The primer concentration which led to the best possible option to amplify 411 bp product was 50 pmole. Cycle numbers did not appear to be critical. All combinations (35, 40 and 45 cycles) produced visible amounts of PCR products (Figure 4B). It was previously reported that sensitivity and specificity of this technique are usually improved by adjustment of concentration of certain ions or nucleotides, by careful design of primers or by properly selecting the cycling conditions (annealing temperature, primer and template concentrations) (Saiki 1989; Innis and Gelfand 1990). Determination of the nucleotide sequences of the fragments amplified from the infected sweet potato plants confirmed their relationship to SPFMV and found to have identities of 95-98% at the nucleotide levels, when compared to published sequences on Gene Bank (data not shown).

A rapid and efficient one step RT-PCR protocol was developed using two 22 bp primers which amplified a 411 bp region of SPFMV coat protein gene. This method yielded a specific fragment which could be visualized by agarose gel electrophoresis and the viral nature of the PCR fragment was confirmed by sequencing. Furthermore, in this protocol, crude nucleic acid from infected sweet potato plants was used. This simplified protocol of nucleic acid extraction was the easiest method of sample preparation compared to the other method

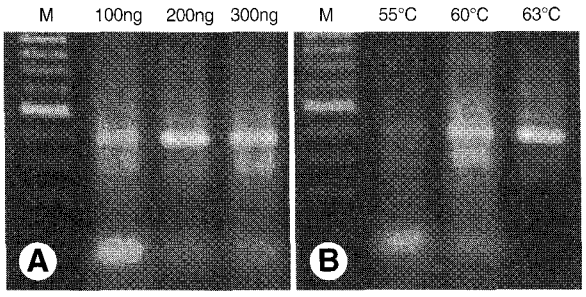

Figure 3. A comparison of the effect of different crude nucleic acid concentrations (A) and annealing temperatures (B) on the RT-PCR reaction. Ethidium bromide-stained agarose gel of RT-PCR products using nucleic acid extracted from SPFMV infected sweet potato plants are shown. The arrow indicates the position of the 411 bp PCR product. Lane M, 100 bp DNA ladders.

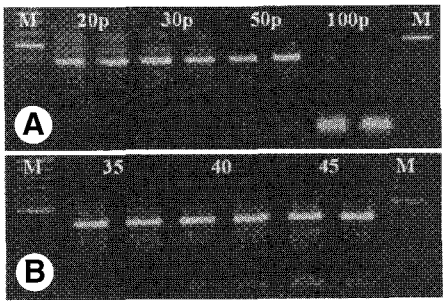

Figure 4. A comparison of the effect of primer concentrations (A) and different reaction cycles (B) on the RT-PCR reaction. Ethidium bromide-stained agarose gel of RT-PCR products using nucleic acid extracted form SPFMV infected sweet potato plants are shown here. The arrow indicates the position of the 411 bp PCR product. Lane M, 100 bp DNA ladders.

available in literature and was suitable for SPFMV detection. There is plenty of evidence that PCR has aided in the detection of a group or family of plant viruses by the use of degenerate primers. In the case of potyviridae, Langeveld et al. (1991) designed degenerate oligonucleotide primers based on the conserved amino acid sequences of the capsid proteins and showed that they amplified DNA fragments of potyviruses in bulb crops. Specific detection of plant viruses, their strains or subtypes is possible by RT-PCR (Griesbach 1995; Hu et al. 1995; Li et al. 1998; Singh and Singh 1998; Jeong et al. 2001). This can be achieved by selecting PCR primers from genome part with the least homology with the other members of a virus group, by using primers based on the viral genome with the highest variability and by selecting a primer pair with the shortest amplified fragment. In this study, the detection procedure was found to be specific not leading to false positives, thus indicating that primer sequences were not detected in the sweet potato genome under these conditions.

Therefore, in this paper, it was shown that the feasibility of detecting SPFMV in sweet potato plants by using a simple efficient one step RT-PCR protocol.

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