Development of Nested PCR-Based Specific Markers for Detection of *Peach Rosette Mosaic Virus* in Plant Quarantine

S. Lee¹ · C. S. Kim¹ · Y. G. Shin² · J. H. Kim³ · Y. S. Kim⁴ · W. H. Jheong⁵

Received: 13 April 2015 / Accepted: 12 August 2015 / Published online: 21 August 2015
© Association of Microbiologists of India 2015

Abstract The *Peach rosette mosaic virus* (PRMV) is a plant pathogen of the genus *Nepovirus*, and has been designated as a controlled quarantine virus in Korea. In this study, a specific reverse transcription (RT)-PCR marker set, nested PCR marker set, and modified-plasmid positive control were developed to promptly and accurately diagnose PRMV at plant-quarantine sites. The final selected PRMV-specific RT-PCR marker was PRMV-N10/C70 (967 bp), and the nested PCR product of 419 bp was finally amplified. The modified-plasmid positive control, in which the *SalI* restriction-enzyme region (GTCGAC) was inserted, verified PRMV contamination in a comparison with

the control, enabling a more accurate diagnosis. It is expected that the developed method will continuously contribute to the plant-quarantine process in Korea.

Keywords Nested PCR · *Peach rosette mosaic virus* · PRMV · Quarantine · RT-PCR

The *Peach rosette mosaic virus* (PRMV) is a plant pathogen of the Group IV positive-sense ssRNA viruses and the genus *Nepovirus* [1]; it was discovered in 1933 by Cation [2]. According to the quarantine-pest data sheet of the crop-protection compendium and the energy policy and planning office, the reported hosts of PRMV include grape (*Vitis labrusca*, *V. vinifera*, and the hybrid *Vitis* spp. in France and the US), peach (*Prunus persica*), peach trees, plum trees, blueberry (*Vaccinium corymbosum*), dandelion, and several weed species (*Rumex crispus*, *Solanum carolinense*, and *Taraxacum officinale*). Crops infected with PRMV generally show blight, senescence, and rosetting. Deformities and discoloration occur on infected fruits and pods, and deformities and abnormal growth occur on leaves and stems [1]. Peach yields decrease by >60 % due to PRMV symptoms and sensitive grape varieties are easily infected [3]. PRMV is an unreported species in Korea, however the possibility of significant economic damage from its emergence in Korea has been raised; therefore, PRMV has been designated as a plant-quarantine-controlled virus to secure quarantine safety [4].

The enzyme-linked immunosorbent assay (ELISA) has been used to diagnose PRMV during the importation of vulnerable crops including *Vitis vinifera*, *Vitis* spp., *Prunus* spp., *P. avium*, *P. serotina*, *Vaccinium* spp., and *T. officinale* [5]. Due to false-positive reactions and the low detection sensitivity of the ELISA [6], though, PCR

S. Lee, Y. G. Shin and J. H. Kim have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s12088-015-0548-2) contains supplementary material, which is available to authorized users.

✉ S. Lee
siwonlee@korea.kr

✉ W. H. Jheong
purify@korea.kr

¹ Environmental Infrastructure Research Department, National Institute of Environmental Research, Incheon 440-170, Korea

² Incheon International Airport Regional Office, Animal and Plant Quarantine Agency, Incheon 440-044, Korea

³ Department of Chemistry, College of Natural Science, Dankook University, Cheonan 330-714, Korea

⁴ Yeongsan River Environment Research Center, National Institute of Environmental Research, Gwangju 500-480, Korea

⁵ Environmental Health Research Department, National Institute of Environmental Research, Incheon 440-170, Korea

analysis has been suggested as an alternative inspection method [5]. However, the existing PCR methods are applied in accordance with diverse conditions and are inconvenient to use at sites where numerous inspections are conducted [7]; therefore, the development of a user-based inspection method with compatible conditions is required. Virus-detection records have increased with the recent development and application of reverse transcription (RT)-PCR, nested PCR, and a modified-plasmid positive control at quarantine sites, as shown by the detection of the *Cherry leaf roll virus* (CLRV) and the *Southern bean mosaic virus* (SBMV), which were not detected by an ELISA; furthermore, the detection of the prohibited *Potato spindle tuber viroid* (PSTVd) in potatoes and the first-ever detection of the *Pea enation mosaic virus* (PEMV) in pea seeds were not reported when only the ELISA was used [1, 5]. And these methods (RT- and nested PCR) are simple and highly sensitive, have recently been studied and established in Korea [8, 9]. Therefore, in this study, we developed RT-PCR- and nested-PCR-specific markers to promptly and accurately diagnose PRMV at plant-quarantine sites; in addition, a modified-plasmid positive control was developed.

The infected samples and total RNA or cDNA from PRMV and 17 reference viruses were collected from relevant organizations such as Bi-One (Korea), Rural Development Administration, and National Academy of Agricultural Science. The RNA-spin™ IIP RNA extraction kit (iNtRON, Korea) was used to extract total RNA from the infected samples. The DNeasy® Plant mini kit (Qiagen, Netherlands) was used to extract total DNA from healthy plants. As PRMV is an RNA virus. So we synthesized cDNA from extracted RNA of PRMV using RT-PCR method. The conditions that were previously reported at quarantine sites in Korea corresponded with RT-PCR, nested PCR (Table 1), and electrophoresis [10].

The RNA complete coding gene that codes a polyprotein gene, and the gene sequences of the *Arabidopsis mosaic virus* (AMV, AY303786), *Raspberry ringspot virus* (RRSV, AY303787), *Tomato black ring virus* (TBRV, AY157993), and *Tobacco ringspot virus* (TRSV, U50869), were collected from the National Center for Biotechnology Information (NCBI) to prepare the specific PRMV marker [10].

Comparison and exclusion of sequence of different viral RNA such as AMV, RRSV, TBRV and TRSV is essential for the design of PRMV specific marker. PRMV specific marker was designed based on AF016626 sequences (PRMV RNA1 polyprotein gene, complete cds) from the NCBI. A multiple alignment of the sequences was performed using BioEdit ver. 7.1.3 [11], and the PRMV-specific markers were searched using the DNAMAN DNA-analysis software package ver. 6.0 (Lynnon Biosoft, Canada), at a marker Tm value of 51–59 °C (with a 5 °C maximum difference for each marker) and DNA G+C content of 45–55 % [8, 12]; as a result, 17 PRMV-specific markers were searched (nine forward and eight reverse). Twenty-one combinations capable of RT-PCR amplification were designed by combining the searched markers, and the set that was most appropriate for the conditions used at quarantine sites was finally selected.

A previously reported method was used to select the RT-PCR and nested PCR markers [7–9, 13], consisting of the following steps: (1) The specific selection was conducted by performing the RT-PCR using total RNA, including PRMV as the template, in order to exclude non-specific binding of the other RNA at total RNA. (2) Eight viruses that were taxonomically similar to PRMV were collected for the first selected set of RT-PCR-marker combinations (*Arabidopsis mosaic virus* [ArMV], *Cherry leaf roll virus* [CLRV], *Cacao necrosis virus* [CNV], *Grapevine fanleaf virus* [GFLV], *Raspberry ringspot virus* [RRSV], *Tomato black ring virus* [TBRV], *Tomato ringspot virus* [ToRSV] and *Tobacco ringspot virus* [TRSV]). Then, the RT-PCR was performed using each extracted total RNA as the template, and the marker combinations that formed non-specific bands were excluded. (3) Nine viruses that can infect a PRMV-vulnerable host were collected for the second selected set of RT-PCR-marker combinations (*Alfalfa mosaic virus* [AMV], *Arracacha virus B* [AVB], *Broad bean wilt virus 2* [BBWV2], *Cucumber green mottle mosaic virus* [CGMMV], *Prune dwarf virus* [PDV], *Prunus necrotic ringspot virus* [PNRSV], *Strawberry latent ringspot virus* [SLRSV], *Tobacco mosaic virus* [TMV] and *Tobacco necrosis virus* [TNV]). Then, the RT-PCR was performed using each extracted total RNA as the template,

Table 1 Condition of RT-PCR and Nested PCR

Step	Temp. (°C)	Time	Cycles	Remark
Reverse transcription	42	30 min	–	Only RT-PCR
Initial denaturation	95	10 min	–	RT-PCR and nested PCR
Denaturation	95	45 s	35	
Annealing	55	1 min		
Extension	72	1 min		
Final extension	72	5 min	–	

RT-PCR and nested PCR conditions of plant quarantine viruses are same condition in Korea

and marker combinations that formed non-specific bands were excluded. (4) PCR was performed using the genomic DNA from goosefoot, peach, globe amaranth, plum, and grape, which are the PRMV hosts for the third selected set of RT-PCR-marker combinations. The marker combinations that formed non-specific bands were excluded. A final RT-PCR combination was selected after the size and location of the amplification product were considered, and the detection sensitivity was determined. (5) A final nested PCR combination was determined through the design and PCR amplification of the nested PCR combination, and was based on the selected RT-PCR product.

The specific detection bands of PRMV were compared using the 21 RT-PCR-marker sets (Supplementary Table S1). The first selection was broadly performed within the RT-PCR product size of 400–1000 bp, and six sets (1, 2, 4, 9, 1, and 16) were selected (Supplementary Fig. S1). We then applied the first six selected sets of the eight-relationship reference-virus strains that were used to examine the PRMV specificity for the positive control, and a non-specific band was analyzed in set 16; therefore, sets 1, 2, 4, 9 and 12 were selected (Supplementary Fig. S2a). Nine host-related viruses were analyzed for the five selected marker sets. The results show that a non-specific band was analyzed for marker set 4, whereas non-specific bands did not form in sets 1, 2, 9 and 12, confirming PRMV marker specificity (Supplementary Fig. S2b). The specificity was reanalyzed, however, using the genomic DNA of each plant body as the template, based on the possibility that PRMV could occur in goosefoot, peach, globe amaranth, plum, or grape. The results of the re-analysis, for which the third of the four selected marker sets was used, show that a non-specific band formed in marker set 9, and it was excluded from the marker sets for the PRMV analysis (Supplementary Fig. S2c). Finally, we selected specific primer sets 1 and 2 in PRMV. Those sets were specific amplification in PRMV and amplification of other viruses and DNA of healthy host were not observed. The PCR information of specific primer sets (Table 2) is summarized.

Quarantine confirmation at plant-quarantine sites is decided through sequencing after a nested PCR analysis. Sets 1 and 2, which were >500 bp, were therefore selected for the RT-PCR products, considering the size of the final nested PCR product, and the detection sensitivity was determined. Marker sets 1 and 2, which were selected in the specificity analysis, were analyzed for the detection-sensitivity experiment, based on a tenfold dilution (10^0 to 10^{-8}) of total RNA including PRMV (53 µg/µl). The results show that set 1 formed a specific band down to the 10^{-2} dilution, and set 2 formed a specific band down to the 10^{-5} dilution. Marker set 2 was therefore appropriate for high-sensitivity PRMV detection and was selected as the final RT-PCR marker, while set 1 was selected as the reserve set (Supplementary Fig. S3). Based on the final selected RT-PCR marker set 2, PRMV_N20/C80 was combined to select the nested PCR marker, and PRMV_N20/C85 was designed and combined for reserve set 1. The RT-PCR amplified the 679 bp product, using a 100-fold diluted template, and the nested PCR marker set 1 (285 bp) was also amplified (Fig. 1). Set 2 (967 bp) was selected as the final RT-PCR marker for the PRMV analysis, and the 419 bp product was selected as the nested PCR amplification product (Fig. 1; Table 2).

The products of the RT-PCR sets 1 and 2 were amplified using both total RNA and the PRMV template to prepare the modified-plasmid positive control. A pGEM®-T Easy vector (Promega, USA) was used to perform the cloning, with the amplified product serving as the insert DNA. Then, six sequences (GTCGAC) that can react with the *SalI* restriction enzyme were inserted inside the set 2 nested-PCR-product region using a site-directed mutagenesis kit (Enzynomics, Korea) [14]. The two bands were confirmed as cut based on five repeat experiments that were performed on the restriction enzyme. The sequencing results confirmed the insertion of GTCGAC. If a sample was contaminated from the modified-plasmid positive control prepared in this study, two bands (132 bp and 293 bp) formed when the amplification product was treated

Table 2 Final selected reserve marker set for specific detection of *Peach rosette mosaic virus*

Primer			Sequences (5' → 3')	Length (bp)	Product size (bp)
Set	PCR	Name			
Finally selective	RT	PRMVN10	CCAAACAACCTTACAGGG	18	967
		PRMVC70	TACGAGGATGTCAGGTAGCG	20	
	Nested	PRMVN20	TCTGTGGGATTGTTGGGAGT	18	419
		PRMVC80	TAAACGCACACCTCCGTAT	19	
Reserve set	RT	PRMVN10	CCAAACAACCTTACAGGG	18	679
		PRMVC80	TAAACGCACACCTCCGTAT	19	
	Nested	PRMVN20	TCTGTGGGATTGTTGGGAGT	18	285
		PRMVC85	ATGGGCACACAGAGCAAT	18

Fig. 1 RT-PCR and nested PCR products for specific detecting *Peach rosette mosaic virus*. Lane M, 100 bp step DNA Ladder marker (Genepia, Korea); lane 1 reserve RT-PCR marker set (PRMV–N10/C80, 679 bp); lane 2 nested PCR marker set of reserve RT-PCR (PRMV–N20/C85, 285 bp); lane 3, finally selective RT-PCR marker set (PRMV–N10/C70, 969); lane 4 nested PCR marker set of finally selected RT-PCR (PRMV–N20/C80, 419 bp)

with a restriction enzyme, and the insertion of six sequences was expected based on the sequencing results (Supplementary Fig. S4).

Recently, various plants have been imported into Korea that seeds have many latent pathogens and are difficult to inspect [5]. The RT-PCR-marker sets, the nested PCR marker sets, and the modified-plasmid positive control were used to promptly and accurately diagnose PRMV, which is an unreported controlled-quarantine virus in Korea. Previously studies, we have been developed fast and accurately diagnosis system that is expected and detected to viruses [15–17]. The developed method was applied to quarantine sites, and it is expected that it will contribute to plant-quarantine processes in Korea.

Acknowledgments This study was conducted with support from the Animal and Plant Quarantine Agency in 2009–2011.

References

- Lee S (2013) A study of molecular biological detection methods for seed-transmitted viruses in quarantine. Dissertation, Dankook University
- Cation D (1933) An infectious rosette on peach trees. Mich Agric Exp Stn Q Bull 16:79–84. doi:[10.1016/0076-6879\(92\)16027-H](https://doi.org/10.1016/0076-6879(92)16027-H)
- Ramsdell DC, Gillett JM, Bird GW (1995) Susceptibility of American grapevine scion cultivars and French hybrid rootstock and scion cultivars to infection by *peach rosette mosaic nepo-virus*. Plant Dis 79:154–157. doi:[10.1094/PD-79-0154](https://doi.org/10.1094/PD-79-0154)
- Animal, Plant and Fisheries Quarantine and Inspection Agency (2013) List of plant quarantine viruses in Korea in newly revised in 2013. Res Plant Dis 19:67–75. doi:[10.5423/RPD.2013.19.1.067](https://doi.org/10.5423/RPD.2013.19.1.067)
- Shin YG (2009) An advanced quarantine system for the inspection of seed-borne viruses in Korea. Dissertation, Kyungpook National University
- Caruso P, Bertolini E, Cambra M, López MM (2003) A new and sensitive co-operational polymerase chain reaction for rapid detection of *Ralstonia solanacearum* in water. J Microbiol Methods 55:257–272. doi:[10.1016/S0167-7012\(03\)00161-1](https://doi.org/10.1016/S0167-7012(03)00161-1)
- Lee S, Kang EH, Heo NY, Kim SM, Kim YJ, Shin YG (2013) Detection of *Carnation necrotic fleck virus* and *Carnation ring-spot virus* using RT-PCR. Res Plant Dis 19:36–44. doi:[10.5423/RPD.2013.19.1.036](https://doi.org/10.5423/RPD.2013.19.1.036)
- Lee S, Kang EH, Chu YM, Shin YG, Ahn TY (2013) Development of PCR diagnosis system for plant quarantine seed-borne wheat streak mosaic virus. Korean J Microbiol 49:112–117. doi:[10.7845/kjm.2013.3013](https://doi.org/10.7845/kjm.2013.3013)
- Lee S, Kang EH, Shin YG, Lee SH (2013) Development of RT-PCR and nested PCR for detection of four quarantine plant viruses belonging to *Nepovirus*. Res Plant Dis 19:220–225. doi:[10.5423/RPD.2013.19.3.220](https://doi.org/10.5423/RPD.2013.19.3.220)
- Lee S, Cha M, Kim SM, Heo NY, Shin YG, Lee SH (2014) Development of nucleotide primers for diagnostic RT-PCR and nested PCR detection of three seed-transmitted viruses (CRLV, SpLV and WCIMV) in quarantine. J Agric Life Sci 48:75–83. doi:[10.14397/jals.2014.48.3.75](https://doi.org/10.14397/jals.2014.48.3.75)
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98
- Pan YB, Burner DM, Legendre BL (2000) An assessment of the phylogenetic relationship among sugarcane and related taxa based on the nucleotide sequence of 5S rRNA intergenic spacers. Genetica 108:285–295. doi:[10.1023/A:1004191625603](https://doi.org/10.1023/A:1004191625603)
- Shin YG, Rho JY (2014) Development of a PCR diagnostic system for Iris yellow spot tospovirus in quarantine. Plant Pathol J 30:440–444. doi:[10.5423/PPJ.NT.06.2014.0052](https://doi.org/10.5423/PPJ.NT.06.2014.0052)
- Nelson M, McClelland M (1992) Use of DNA methyltransferase/endonuclease enzyme combinations for megabase mapping of chromosomes. Methods Enzymol 216:279–303. doi:[10.1016/0076-6879](https://doi.org/10.1016/0076-6879)
- Lee S, Shin YG (2014) Development and practical use of RT-PCR for seed-transmitted prune dwarf virus in quarantine. Plant Pathol J 30:178–182. doi:[10.5423/PPJ.NT.10.2013.0099](https://doi.org/10.5423/PPJ.NT.10.2013.0099)
- Lee S, Min BD, Lee JY, Shin YG, Lee SH, Jheong WH (2015) Development of diagnostic PCR system for three seed transmitted quarantine viruses associated with cucurbitaceae. Microbiol Biotechnol Lett 43:79–83. doi:[10.4014/mb.1410.10008](https://doi.org/10.4014/mb.1410.10008)
- Lee S, Lee G, Choi IC, Rho JY (2015) Development of PCR diagnostic system for detection of the seed-transmitted tobacco ringspot virus in quarantine. Indian J Microbiol 55:231–233. doi:[10.1007/s12088-015-0518-8](https://doi.org/10.1007/s12088-015-0518-8)