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SHORT COMMUNICATION

Development of PCR Diagnostic System for Detection of the Seed-Transmitted *Tobacco Ringspot Virus* in Quarantine

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Abstract *Tobacco ringspot virus* (TRSV) is a plant quarantine virus in Korea. As such, a TRSV examination is conducted when importing various crops. In this study, RT-PCR and nested PCR systems for TRSV detection in quarantine sites, and the modified-positive control plasmid for proving laboratory contamination and false positive reactions were developed. The developed diagnostic system was used to detect TRSV in the quarantine site. It revealed that from 2012 to August 2014, a total of 12 cases were detected in imported various crops. The system is expected to continue contributing to TRSV detection in plant quarantine.

Keywords *Tobacco ringspot virus* · TRSV · RT-PCR · Nested PCR · Quarantine

Tobacco ringspot virus (TRSV) is a plant pathogen classified as a Group IV (+) sense ssRNA virus in family Secoviridae, genus *Nepovirus* [1]. They have a very wide range of host plants that is transmitted seeds and nematodes [2]. TRSV is the non-reported controlled quarantine virus in Korea. Thus, when importing potatoes, mung beans, soy beans, melons, Oriental melons, lettuce, sweet cherries, geranium genus, globe amaranths, and petunia, a TRSV examination is conducted [3].

To diagnosis of TRSV has long been using the enzyme-linked immunosorbent assay (ELISA) method in Korea [4]. ELISA was first used in 1977 to detect plant viruses [5]. However, its false positive reaction makes it difficult to conduct a precise diagnosis and quarantine examination, so methods with a higher detection capability are needed to detect a very tiny amount of viruses in a few infected seeds among a huge number of seeds [6, 7]. In this study, the polymerase chain reaction (PCR) method, which had been found in a study to have a high degree of safety and was thoroughly studied recently as a quarantine examination method, was reviewed [8–12]. However, the following important problems were found. First, it requires different applicable temperatures, which makes it inconvenient to use in a quarantine site where various pathogens must be simultaneously examined. Second, when more than two kinds of viruses must be examined in the same seed, ELISA and PCR have to be applied separately, thereby wasting labor and manpower, and causing inconvenience in the quarantine site. Third, if the positive control sample group is not secured, PCR test errors and amplification or non-amplification are difficult to accurately assess [13]. Moreover, since the positive control group sample is infected with the virus, it may be difficult to distribute domestically and import [14]. Therefore, in this study, both RT-PCR and nested PCR methods were developed to accurately diagnose the plant quarantine seed-transmitted TRSV, which may infect diverse imported seeds. To prove laboratory contamination and a false positive reaction, the modified-positive control plasmid was manufactured for use as the positive control group in the PCR examination system. In addition, this paper reports the results of the

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TRSV quarantine examination conducted from 2012 to the first half of 2014, using the developed examination method.

The affected samples, and the RNA or cDNA of the targeted TRSV as well as of 17 kinds of reference viruses, were purchased after the approval of the products banned for import (Bione, Korea), or were gathered from related institutions such as the Rural Development Administration. Furthermore, among the crops expected to be infected with TRSV, healthy potatoes, melons, lettuce, and soy beans were gathered.

To design TRSV-specific primers, the base sequences of TRSV strains and references were gathered from the National Center for Biotechnology Information. The gathered base sequences of the viruses were arranged using BioEdit [15], before the DNAMAN software package was used and the species-specific base sequences were explored at the annealing temperature range of 51–59 °C [16, 17], which revealed 10 forward primers and 12 reverse primers (Supplementary Fig. S1; Supplementary Table S1). The designed primers were synthesized by Bioneer (Korea), and the species-specific primers were combined to yield 48 combinations that could amplify the 148–1,060 bp products and the RT-PCR (Supplementary Table S2).

RNA was extracted from the affected samples, cDNA was synthesized, DNA was extracted from healthy plants, and RT-PCR and electrophoresis were performed as previously described [5, 18].

To select the combinations of the TRSV RT-PCR primers, the following five-stage selection procedure was conducted [2]. A particular band was primarily analyzed. The analysis revealed that the particular band was formed in all the 48 applied primer sets. Among them, considering the clear formation of the particular band and the location of the amplified products, 11 primer sets (set 2, 3, 7, 8, 9, 13, 14, 20, 21, 26, and 48) were selected (Supplementary Fig. S2). Related viruses were applied to these 11 primarily selected RT-PCR primer sets to determine the TRSV specificity. It revealed that sets 9, 13, 14, and 48 formed a non-specific band; set 26 formed too small products; and set 2, 3, 7, 8, 20, and 21 did not form a band (Supplementary Fig. S3). The 11 reference viruses that could infect the possibly TRSV-infected hosts in the six secondarily selected primer sets were subjected to a non-specific analysis. The analysis revealed that set 7 and 8 formed a non-specific band, and set 2, 3, 20, and 21 did not form a band against the related viruses. Thus, the specificity of TRSV was confirmed, so the primer sets were deemed suitable (Supplementary Fig. S4). With the greater possibility that TRSV may infect potatoes, melons, lettuce, and soy beans, the specificity of the four selected third primer sets was analyzed using individual genomic DNAs as the templates. The analysis revealed that no non-specific band was formed, which confirmed that the four primer sets were all TRSV-specific primer sets (Supplementary Fig. S5). However, the sensitivity test of individual primer sets revealed that set 2, 3, 20, and 21 had a detection sensitivity of 10^{-6}, 10^{-3}, 10^{-7}, and 10^{-5}, respectively. Thus, sets 2 and 20 were selected as the final TRSV RT-PCR primer sets (Supplementary Fig. S6A).

Furthermore, to conduct the nested PCR analysis that enabled the re-amplification of the TRSV RT-PCR products, primers were combined. For the RT-PCR primer set 2, four nested PCR combinations were formed; and for set 20, two nested PCR combinations were also formed. The nested PCR primers were evaluated. The six nested PCR combinations all formed a specific band. However, their sizes and base sequences were compared with those of the RT-PCR products, which revealed that TRSV-N12/TRSV-C90 (415 bp) and TRSV-N40/TRSV-C70 (234 bp) showed an appropriate size for identification and formed a clear and bright band. They were thus evaluated as the nested PCR primer sets (Supplementary Fig. S6B). Based on these results, set 2 (691 bp) and set 20 (580 bp) were finally selected as the RT-PCR primer combinations for TRSV analysis, and the individual nested-PCR-amplified products were confirmed as 415 and 236 bp (Fig. 1; Table 1).

To manufacture the modified-positive control plasmid for use in the TRSV RT-PCR and nested PCR diagnosis systems was performed as previously described [18, 19]. To confirm the inserted gene, TRSV RT-PCR set 2 was amplified using the manufactured modified-positive control plasmid as the template DNA. With the amplified product

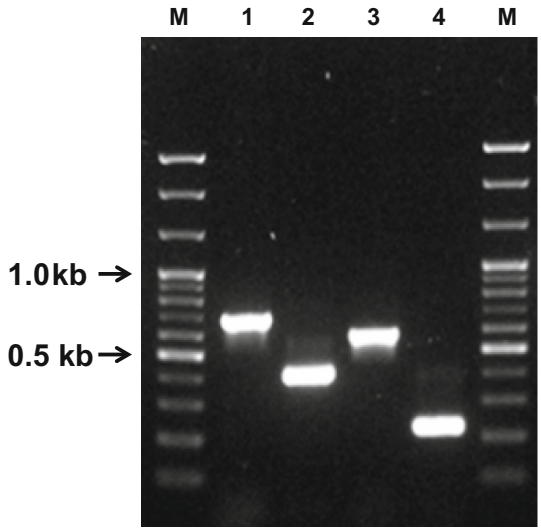

Fig. 1 Result of the finally selected RT-PCR and nested PCR primer sets for the detection of the *Tobacco ringspot virus* M, 100 bp step DNA Ladder maker (Genepia, Korea); lane 1, TRSV finally selected RT-PCR primer set 2 (691 bp); lane 2, TRSV nested-PCR product from RT-PCR primer set 2 (TRSVN12/C90, 415 bp); lane 3, TRSV RT-PCR primer set 20 (581 bp); lane 4, TRSV nested-PCR product from RT-PCR primer set 20 (TRSVN40/C70, 234 bp)

Table 1 Information on the finally selected RT-PCR and nested PCR primer sets for the detection of the *Tobacco ringspot virus*

Primer			Sequence (5′ → 3′)	Length (mer)	Band size (bp)
Set	PCR	Name			
2	RT	TRSVN10	GTTGTTCCCGATCCCACTTGTTG	23	691
		TRSVC80	TAACTGCCCGGGAATATGAAATGG	24	
	Nested	TRSVN12	CCCAAAGATGCGAAGAAAGGAA	22	415
		TRSVC90	GCCAATCAGCAGCCATCGTC	20	
20	RT	TRSVN30	TCCGAGATGTTTGAACTGCCTACC	24	581
		TRSC30	TGGCCATCTCCGTGCATTATCTGA	24	
	Nested	TRSVN40	ACTGAAGAAGGGTTTGGTAGACT	23	234
		TRSVC70	CTTCCGCTTATAGTGCCAGACCA	23	

as the template, the band was confirmed using TRSV-N12/TRSV-C90. The amplified product was made to undergo reaction using the restriction enzyme *Xho* I, which revealed two bands (Supplementary Fig. S7).

The proposed TRSV PCR diagnosis system has been applied to the quarantine site since 2012. According to Pest Information System, from January 2012 to August 2014, a total of 12 cases were detected in imported plants and abolished or returned. The proposed PCR quarantine diagnosis system is expected to continue to speedily and accurately detect TRSV in Korea's quarantine sites, and thereby, to contribute significantly to the quarantine activities.

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