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RT-PCR Detection of Five Quarantine Plant RNA Viruses Belonging to Poty- and Tospoviruses

Jong-Seung Lee[1], Won Kyong Cho[1], Hong-Soo Choi[2] and Kook-Hyung Kim[1]*

[1]*Department of Agricultural Biotechnology and Plant Breeding and Genomics Institute, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea*

[2]*Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Korea*

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In order to detect quarantine plant viruses, we developed reverse transcription-polymerase chain reaction (RT-PCR) primer pairs for five single-stranded (ss) plant RNA viruses that are not currently reported in Korea but could be potential harmful plant viral pathogens. Three viruses such as *Chilli veinal mottle virus* (ChiVMV), *Colombian datura virus* (CDV), and *Tobacco etch virus* (TEV) belong to the genus *Potyvirus* while *Chrysanthemum stem necrosis virus* (CSNV) and *Iris yellow spot virus* (IYSV) are members of the genus *Tospovirus*. To design RT-PCR primers, we used reported gene sequences corresponding to the capsid protein and polyprotein for ChiVMV, CDV, and TEV while using nucleocapsid protein regions for CSNV and IYSV. At least two different primer pairs were designed for each virus. Fifteen out of 16 primer pairs were successfully applied in detection of individual quarantine virus with high specificity and efficiency. Taken together, this study provides a rapid and useful protocol for detection of five quarantine viruses.

Keywords **:** *Potyvirus*, Quarantine, RT-PCR, ssRNA virus, *Tospvirus*

In recent years, global climate change impacts on our enviromental systems including agriculture and ecosystem. Increased average temperature affects survival rates of insect vectors which are the main factors promoting incidence and spread of plant viral diseases (Garrett et al., 2006). The climiate of the Korean pennisula becomes warmer than before and several unexpected diseases led to dramatic economic losses in many important crops, vegetables, and fruits (Choi et al., 2009). In addition, a large amount of agricultural products are being imported from the world to stabilize prices and this will be accelarated by Free Trade Agreement (FTA) between Korea and other countries including the United States and European Union. Eventually, the possibility of introduction of foreign plant viruses via imported plants and foods becomes increasing. Moreover, the changed chlimate in Korea will be suitable for foreign viruses to replicate and spread in new host plants (Elena et al., 2011). For these reasons, the National Plant Quarantine Service (NPQS) in Korea needs to strengthen plant quarantine. To control inflow of quarantine plant viruses, effective monitoring methods are required. Of known techniques, reverse transcription-polymerase chain reaction (RT-PCR) is the rapid tool with highly specific and sensitive for detection of individual virus (Lee et al., 2011; Park and Kim, 2004). In a close cooperation with the NPQS, we are currently developing RT-PCR based detection system for major quarantine plant viruses and have reported RT-PCR detection methods for quarantine plant viruses infecting fruit trees and the family *Cucurbitaceae* or *Solanaceae* (Lee et al., 2011; Park and Kim, 2004).

In a series of detecting quarantine plant viruses, five additional single-strand RNA viruses causing significant economic problems to many vegetables and flowering plants were further chosen in this study. These viruses include *Chilli veinal mottle virus* (ChiVMV; Hwang et al., 2009), *Colombian datura virus* (CDV; Salamon and Palkovics, 2005), *Tobacco etch virus* (TEV; Carrasco et al., 2007), *Chrysanthemum stem necrosis virus* (CSNV; Bezerra et al., 1999), and *Iris yellow spot virus* (IYSV; Pappu et al., 2006) (Table 1). ChiVMV, CDV and TEV are positive-strand RNA viruses and consist of one RNA segment belonging to the genus *Potyvirus*, family *Potyviridae* (Table 1). In contrast, CSNV and IYSV are negative-strand RNA viruses and composed of three RNA segments belonging to the genus *Tospovirus*, family *Bunyaviridae* (Table 1).

All three potyviruses can infect plants in the *Solanaceae* family (Table 1). For instance, ChiVMV is one of major virus infecting chilli pepper causing mosaic, mottling, leaf distortion, vein etching, yellowing, and stunting symptoms.

*Corresponding author.
Phone) +82-2-880-4677, FAX) +82-2-873-2317
E-mail) kookkim@snu.ac.kr

Since the first identification of CDV from Brugmansia in Colombia in the late 1960s, it is quickly spread from South America such as Colombia, Ecuador, and Bolivia to Europe by imported plant materials. CDV infects angel trumpets (*Brugmansia* spp.), cape gooseberry (*Physalis peruviana*), and pepino (*Solanum muricatum*) in Hungary (Salamon and Palkovics, 2005). TEV is one of well characterized plant virus in the genus *Potyvirus* and many plants including pepper, tomato, and tobacco species in the *Solanaceae* can be infected by TEV. TEV causes mosaic and dark green vain banding in infected pepper leaves and cause stunted and misshapened fruit formation. CSNV has been firstly reported in Netherlands and Brazil where chrysanthemum is largely cultivated. Symptoms of CSNV in chrysanthemum are necrotic local lesions with concentric yellow spots on leaves leading to the death of most plants (Bezerra et al., 1999). IYSV infects mostly *Allium* species like onion, ornamentals like iris, and lisianthus (Diaz-Montano et al., 2011). In particular, the initial identification of IYSV in Brazil, IYSV is one of serious viral diseases in many onion producing countries. Symptoms of IYSV composed of yellow or straw colored diamond shaped lesions on the scape or leaves in onion plants (Diaz-Montano et al., 2011). All three potyviruses including ChiVMV, CDV, and TEV are principally transmitted by the aphid species in a non-persistent manner. They can also be transmitted by mechanical inoculation and grafting but not by seeds. Both CSNV and IYSV are transmitted by the family *Thripidae* including *Frankliniella occidentalis* and *Frankliniella schultzei* for CSNV (Bezerra et al., 1999) and *Thrips tabaci* for IYSV (Diaz-Montano et al., 2011) in a persistent manner.

To design RT-PCR primer pairs, we utilized known available RNA sequences for each virus deposited in National Center for Biotechnology Information (NCBI). In case of ChiVMV, CDV, and TEV, RNA sequences encoding capsid protein (CP) and polyprotein were retrieved for primer design due to high conservancy within the virus species (Table 2). As for the RT-PCR detection of CSNV and IYSV, gene regions corresponding to non-structural and nucleocapsid protein (NCP) genes were used (Table 2). At least two different primer pairs for individual gene were designed and the lengths of primer pairs were varied to distinguish quarantine viruses easily (Table 2). For instance, the lengths of PCR fragments to detect the CP of ChiVMV are 389 bp and 550 bp, and TEV can be detected with fragments ranged from 259 bp and 529 bp. Overall, the size of amplified PCR fragments ranges from 236 bp to 662 bp. The information for RT-PCR primers can be found in Table 2. All primers were synthesized from Bioneer Corp. (Korea).

We imported all five plant ssRNA viruses from DSMZ GmbH, plant virus collection (Braunschweig, Germany). The imported samples were either dried plant materials or frozen total nucleic acids deposited at –20°C. All processes related to the import of quarantine viruses were done after permission from NPQS and all experiments were carried out in restricted area to prevent virus outflow. Total RNAs derived from virus infected plant tissues as well as healthy leaves of *Nicotiana benthamiana* and tomato were prepared by extraction with Trizol Reagent® (Molecular Research Center, Inc., USA) followed by phenol:chloroform:isoamyl-alcohol (25:24:1) extraction and ethanol precipitation according to the previous study (Park and Kim, 2004). The extracted total RNAs were analyzed by agarose gel electrophoresis and subjected to the NanoPhotometer™ (Implen GmbH, Germany) to measure quality and quantity of the RNAs, respectively. As shown Fig. 1A, the quality and quantity of extracted total RNAs were good enough to carry out RT-PCR indicating high quality of isolated RNAs. The cDNAs were synthesized using the extracted total RNAs and PCRs were conducted using the cDNA as a template. Syntheses of cDNAs and RT-PCR reactions were conducted as described previously (Lee et al., 2011). In brief, complementary DNAs (cDNAs) were synthesized by using

Table 1. List of plant viruses assayed for quarantine

Plant virus name	Genus	Family	Type	No. of RNA fragments	Host plants
Colombian datura virus (CDV)	*Potyvirus*	*Potyviridae*	ssRNA (+)-strand	1	Tomato, pepino, tobacco, cape gooseberry, petunia
Chilli veinal mottle virus (ChiVMV)	*Potyvirus*	*Potyviridae*	ssRNA (+)-strand	1	Pepper, tobacco, nightshade
Tobacco etch virus (TEV)	*Potyvirus*	*Potyviridae*	ssRNA (+)-strand	1	Pepper, tomato, tobacco, nightshade, pigweed, ground cherry
Chrysanthemum stem necrosis virus (CSNV)	*Tospovirus*	*Bunyaviridae*	ssRNA (–)-strand	3	Chrysanthemum, tomato
Iris yellow spot virus (IYSV)	*Tospovirus*	*Bunyaviridae*	ssRNA (–)-strand	3	Onions, garlic, leek, iris, lisianthus, jimsonweed, tobacco, redroot pigweed

Table 2. Nucleotide sequences of oligonucleotide primers used for RT-PCR to detect five quarantine plant viruses

Index	Name	Amplified region	Nucleotide accession No.	Primer sequences	Length	Tm (°C)	Expected size	RT-PCR result
1	CDV-CP1_F	capsid protein	FJ821796	5'-GGTGTCTGGACTATGATGGATG-3'	22	52.9	420 bp	Specific
2	CDV-CP1_R	capsid protein	FJ821796	5'-CCTCTAACCTTGACGCACACC-3'	21	55.8	420 bp	Specific
3	CDV-CP2_F	capsid protein	FJ821796	5'-CTGCAGCAATTCGAAACAACAC-3'	22	56.9	281 bp	Specific
4	CDV-CP2_R	capsid protein	FJ821796	5'-GTAAATACTGACTAACGCCCTTCTG-3'	25	54.5	281 bp	Specific
5	CDV-Poly1_F	polyprotein	AB179622	5'-CAAGATCGAGATGTGAATGCTG-3'	22	54.5	662 bp	Specific
6	CDV-Poly1_R	polyprotein	AB179622	5'-GTGTGACGTTCGGTATCCTCTTC-3'	23	56.2	662 bp	Specific
7	CDV-Poly2_F	polyprotein	AB179622	5'-CTGTGCAAGCATCATAGAGTCG-3'	22	54.8	498 bp	Specific
8	CDV-Poly2_R	polyprotein	AB179622	5'-GGTTTGTAGTTCAGCAGATGGTC-3'	23	54.2	498 bp	Specific
9	ChiVMV-CP1_F	capsid protein	NC_005778	5'-CAAGCTCAGCCACAGTCTCGTC-3'	22	58.6	550 bp	Specific
10	ChiVMV-CP1_R	capsid protein	NC_005778	5'-CGCGCTAATGACATATCGGTAAG-3'	23	57.5	550 bp	Specific
11	ChiVMV-CP2_F	capsid protein	NC_005778	5'-CTGAATGGATTGATGGTTTGGTG-3'	23	57.7	389 bp	Specific
12	ChiVMV-CP2_R	capsid protein	NC_005778	5'-GTGCCTACCCTACCGTCCAGTC-3'	22	58.3	389 bp	Specific
13	ChiVMV-Poly1_F	polyprotein	EF213685	5'-CTCTGAGGGGAAGGCACCATAC-3'	22	58.4	443 bp	Specific
14	ChiVMV-Poly1_R	polyprotein	EF213685	5'-CAGGGGCATAATCAAGAAGGTG-3'	22	57.1	443 bp	Specific
15	ChiVMV-Poly2_F	polyprotein	EF213685	5'-GCGGTATGCTTTCGATTTCTATG-3'	23	56.8	404 bp	Specific
16	ChiVMV-Poly2_R	polyprotein	EF213685	5'-GCTAGAACGGGCGGGGATAC-3'	20	59.2	404 bp	Specific
17	TEV-CP1_F	capsid protein	DQ871317	5'-CGCTATGGCCACAAAACTTCAAT-3'	23	58.9	339 bp	Specific
18	TEV-CP1_R	capsid protein	DQ871317	5'-GTCATAATTTGCCTCAGTGTTGG-3'	23	55	339 bp	Specific
19	TEV-CP2_F	capsid protein	DQ871317	5'-CTAAATGGATTTATGGTGTGGTG-3'	23	52.6	391 bp	Specific
20	TEV-CP2_R	capsid protein	DQ871317	5'-CAGTACCCACGTTGCCATCA-3'	20	56.3	391 bp	Specific
21	TEV-Poly1_F	polyprotein	NC_001555	5'-GACCCAAATGAAGGATACGATAAG-3'	24	54.7	259 bp	Specific
22	TEV-Poly1_R	polyprotein	NC_001555	5'-GTCTCTTCGTTGGGTGCTTTAG-3'	22	54.5	259 bp	Specific
23	TEV-Poly2_F	polyprotein	NC_001555	5'-GTGTGCAAAGAAATTCCAGACTC-3'	23	54.3	529 bp	Specific
24	TEV-Poly2_R	polyprotein	NC_001555	5'-CACCACCAATTAACACAGACAAAG-3'	24	55	529 bp	Specific
25	CSNV-NCP1_F	nucleocapsid protein	AB438998	5'-GCGGAATACTCTGCACGACTTG-3'	22	58.4	601 bp	Specific
26	CSNV-NCP1_R	nucleocapsid protein	AB438998	5'-GCTCTTTGTGCTTTGAATCCTG-3'	22	55.4	601 bp	Specific
27	CSNV-NCP2_F	nucleocapsid protein	AB438998	5'-CTTACGGGCTTAGCTTGAATG-3'	21	53.3	300 bp	Specific
28	CSNV-NCP2_R	nucleocapsid protein	AB438998	5'-CTCCTTTCATTCTTGGGTCAC-3'	21	52.4	300 bp	Specific
29	IYSV-NCP1_F	nucleocapsid protein	AF067070	5'-TCTGGTGAGTGCATATGGTTTGA-3'	23	56.7	420 bp	Un-specific
30	IYSV-NCP1_R	nucleocapsid protein	AF067070	5'-CTTGGAGGGATTCTTGGGTTTAG-3'	23	56.9	420 bp	Un-specific
31	IYSV-NCP2_F	nucleocapsid protein	AF067070	5'-GGCGGTCCTCTCATCTTACTG-3'	21	55	236 bp	Specific
32	IYSV-NCP2_R	nucleocapsid protein	AF067070	5'-GAAGTTCCAGGAGTGCATTTAGTC-3'	24	54.9	236 bp	Specific

M-MLV reverse transcriptase (RT) and RT-PCR was performed for 30 cycles using a Peltier Thermal Cycler (MJ Research, USA). Five μl of amplified PCR products were separated on 1% agarose gel by electrophoresis, visualized by staining with 0.5 μg/ml EtBr, and observed under UV illumination.

For first PCR reaction, we used 53°C and 30 sec as an annealing temperature and time. Of designed 16 primer pairs, 15 primer pairs showed successful PCR amplification except IYSV-NCP1 which displayed two amplified PCR products (Fig. 1A). In case of CDV detection, primer pairs for CDV-CP2 and CDV-Poly1 amplified one specific band with high amount of transcripts but the band intensity for CDV-CP1 is relatively weak suggesting that CDV-CP1 primer pair was not optimally bound to target CDV RNA sequences (Fig. 1B).

To optimize annealing temperature for individual primer pair, gradient PCR was conducted at three different temper-

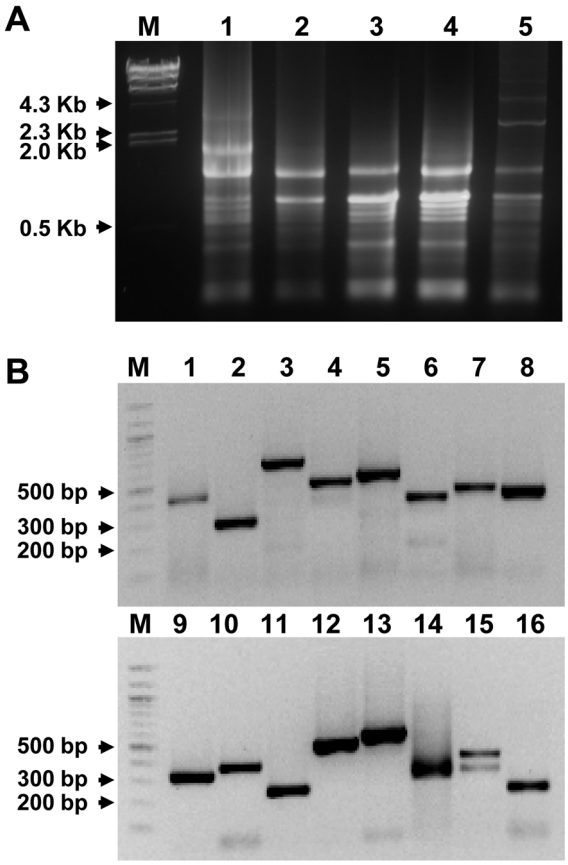

Fig. 1. Isolation of total RNAs and RT-PCR using newly developed primer pairs. (A) Total RNAs extracted from virus infected plant leaves. M indicates lamda DNA molecular marker digested with *Hin*dIII (600 ng/5 μl). Lanes 1–5 represent RNA extracted from CDV, ChiVMV, TEV, CSNV, and IYSV infected samples, respectively. (B) RT-PCR using following primer pairs. Lane 1, CDV-CP1; Lane 2, CDV-CP2; Lane 3, CDV-Poly1; Lane 4, CDV-Poly2; Lane 5, ChiVMV-CP1; Lane 6, ChiVMV-CP2; Lane 7, ChiVMV-Poly1; Lane 8, ChiVMV-Poly2; Lane 9, TEV-CP1; Lane 10, TEV-CP2; Lane 11, TEV-Poly1; Lane 12, TEV-Poly2; Lane 13, CSNV-NCP1; Lane 14, CSNV-NCP2; Lane 15, IYSV-NCP1; Lane 16, IYSV-NCP2.

atures including 52°C, 55°C, and 57°C (Fig. 2). Interestingly, as annealing temperature goes up, the amount of amplified PCR was increased. At 52°C, except three primer-pairs including ChiVMV-CP1, TEV-Poly2, and CSNV-NCP1, the intensity of band was rather weak. Especially, primer pairs for CDV-CP1 and IYSV-NCP2 could hardly amplify PCR products. At both 55°C and 57°C for annealing temperature, we detected additional unspecific bands from CDV-Poly1 and ChiVMV-CP2 primer pairs which might be amplified by binding to other genes in host plants. To test such hypothesis, we extracted total RNAs from leaves of virus free tomato and *N. benthamiana* and synthesized cDNAs. Using the cDNAs derived from virus free plants as templates, we carried out RT-PCR at 55°C. All

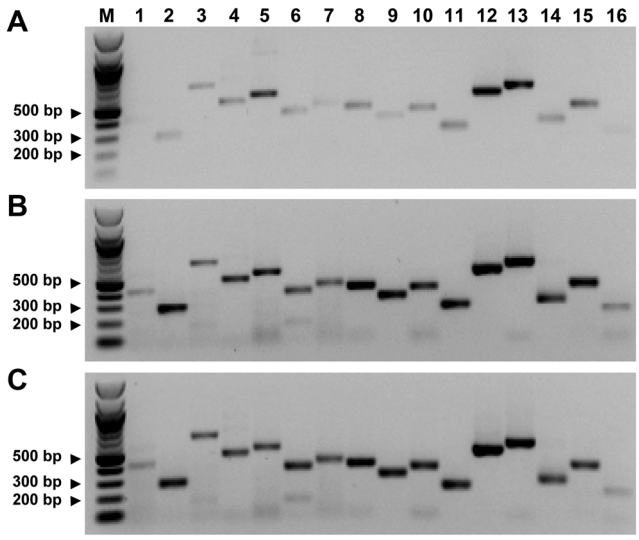

Fig. 2. Gradient RT-PCR to optimize specificity of each primer pair. M indicates 100 bp DNA ladder purchased from NEB, UK. PCR products from three different annealing temperatures including 52°C (A), 55°C (B), and 57°C (C) were shown. Lanes 1, CDV-CP1; 2, CDV-CP2; 3, CDV-Poly1; 4, CDV-Poly2; 5, ChiVMV-CP1; 6, ChiVMV-CP2; 7, ChiVMV-Poly1; 8, ChiVMV-Poly2; 9, TEV-CP1; 10, TEV-CP2; 11, TEV-Poly1; 12, TEV-Poly2; 13, CSNV-NCP1; 14, CSNV-NCP2; 15, IYSV-NCP1; 16, IYSV-NCP2.

primer pairs displayed only one expected band in virus infected samples but not in virus free plant samples (Fig. 3). Thus, it is likely that the amplified unspecific bands were derived not from nonspecific binding of designed primer pairs but from target plant virus. We assume that the designed primer pairs could also bind to not only target sequences but also other nucleotide regions or degraded RNA genomes of the quarantine plant viruses. Nevertheless, we can apply the designed primer pairs to detect quarantine viruses since these primers can amplify expected size DNA fragments as major amplification PCR products. In many case, viral proteins in same genus are highly homologous. Thus, it is likely that the designed primer pairs for CP or polyprotein can amplify other viral genes in different virus species. To reveal such possibility, we performed RT-PCR using heterogous mix of primer pairs and cDNAs. No PCR products were obtained indicating that nucleotide sequences for conserved viral proteins are highly diverse among virus species or genus (data not shown). The majority of imported plants are seeds which might be infected with quarantine plant viruses. Thus, the designed RT-PCR primer pairs could be applied to detect quarantine plant viruses in imported seeds. For that, we are now performing experiments including infection of quarantine viruses into seeds of various host plants and developing techniques to detect viruses in seeds with high sensitivity and simplicity. A previous study demonstrated real time RT-PCR based approach could

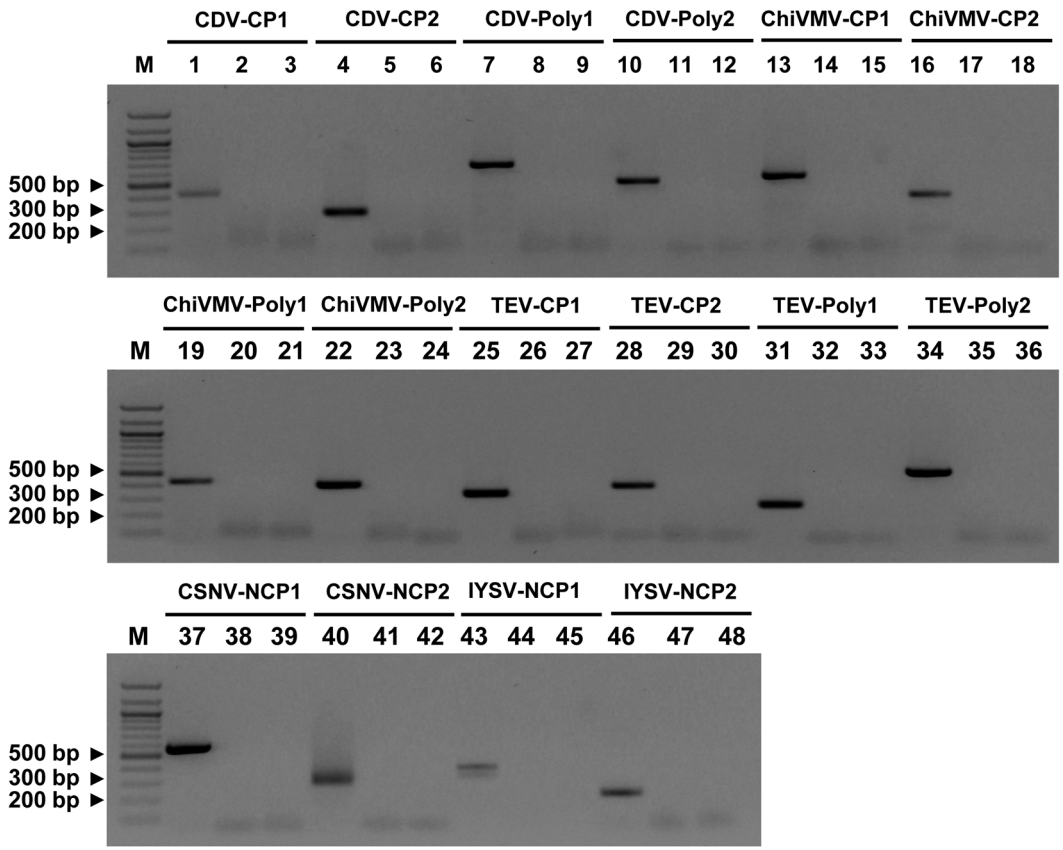

Fig. 3. Specificity of newly designed primers for virus detection by RT-PCR. M indicates 100 bp DNA ladder purchased from NEB, UK. Total RNAs from virus infected (lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, and 46), healthy tomato (lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, and 47) and healthy *N. benthamiana* (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, and 48) leaves were used for RT-PCR analyses.

detect *Maize chlorotic mottle virus* in maize seeds (Zhang et al., 2011).

Although possibility of foreign plant pathogens including viruses, fungi, and bacteria to introduce into Korea is gradually increasing, only a limited number of studies have reported diagnostic system to detect them. For instance, three recent studies developed virus specific primers for RT-PCR based detection (Lee et al., 2004; Lee et al., 2011; Park and Kim, 2004). The first study developed primer pairs to detect seed-transmissible viruses (Lee et al., 2004) while the other studies demonstrated the specificity of designed primer pairs to detect five viruses infecting vegetables and three viruses associated with fruit trees like apple and cherry (Lee et al., 2011; Park and Kim, 2004). In this study, a set of new primers for the detection of five non-reported plant viruses were designed and employed for RT-PCR detection. The specificity of each newly designed primer pairs was also evaluated by RT-PCR assay. We identified at least one primer pair which can amplify one specific band for individual plant virus. Moreover, we tested various annealing temperature for PCR and high annealing temperature often led to increase the amount of PCR products and specificity. As compared to our previous study (Lee et al., 2011), we provide more primer pairs to detect each virus and the specificity of designed primer pairs were dramatically increased with 93.75%. The successful application of newly developed RT-PCR primer pairs will be usefully used to detect five quarantine plant viruses.

So far, except RT-PCR, various methods are being applied to detect viruses such as antibodies based enzyme-linked immunosorbent assay (ELISA) and electron microscopy. For instance, antibodies generated from recombinant proteins or synthetic polypeptides could successfully detect *Rice stripe virus* in rice and weed plants (Lian et al., 2011). Moreover, antibody based immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) method has shown to be more sensitive method than ELISA or dot-blot immunobinding assay (Sreenivasulu and Sai Gopal, 2010). It is known that many viruses are transmitted by seeds. Generally, to detect viruses in seeds, seeds were first planted in a controlled green house and virus symptoms could be observed. However, such approach requires time and labor.

Therefore, direct RT-PCR detection method which is precise and efficient could complement such problem. In addition, real-time PCR assay is recently regarded as the next favorable technique because of its high sensitivity (Harper et al., 2011; Zhang et al., 2011). A previous study has shown that 4 fg of total RNA or 25 copies of RNA transcripts are high enough to detect virus and sensitivity of this method seems to be ten-fold higher than other RT-PCR gel electrophoresis approach (Zhang et al., 2011). Therefore, it could be of interest to develop detection system based on real time RT-PCR in near future. In summary, this study provides a useful protocol to detect five ssRNA viruses rapidly for quarantine purpose.

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