Plant Pathol. J. 20(4) : 302-307 (2004)

Development of Molecular Detection of Three Species of Seed-Transmissible Viruses Useful for Plant Quarantine

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(Received on January 28, 2004; Accepted on September 10, 2004)

Three pairs of specific primers were developed for rapid and precise RT-PCR detection of three seed-transmissible viruses, namely *Peanut clump virus* (PCV, *Pecluvirus*), *White clover mosaic virus* (WCIMV, *Potexvirus*) and *Carrot red leaf virus* (CaRLV, *Luteovirus*). Each primer set was found in conserved region through multiple sequence alignment in the DNAMAN. Total nucleic acids extracted from PCV-, WCMV-, and CaRLV-infected seeds and healthy plants were used for RT-PCR detection using each virus-specific primer. Sizes of PCV, WCIMV, and CaRLV PCR products were 617bp (PCV-uni5 and PCV-uni3 primers), 561bp (WCMV-CP5 and WCMV-CP3 primers), and 626bp (CL1-UP and CL2-DN primers); which corresponded to the target sizes. Nucleotides sequences of each amplified cDNA were confirmed which belonged to the original virus. This study suggests that these virus-specific primer sets can specifically amplify viral sequences in infected seeds. Thus, they can be used for specific detection of three viruses (PCV, WCMV and CaRLV) from imported seed samples for plant quarantine service.

Keywords : *Carrot red leaf virus* (CaRLV), detection, *Peanut clump virus* (PCV), plant quarantine, RT-PCR, seed transmission, *White clover mosaic virus* (WCIMV)

Seed transmission provides a very effective means of introducing virus into a crop at an early stage; thus, giving randomized foci of primary infection throughout the planting (Hull, 2002) are prerequisite for control of seed-transmisible viruses. Approximately 20% of plant viruses are transmitted from generation to seed (Wang and Maule, 1994), and yet very little is known about the mechanism involved (Maule and Wang, 1996; Mink, 1993). Generally, seed-borne virus may be present for a long time in seed and can be spread worldwide. *Peanut clump virus* (PCV, *Pecluvirus*), *White clover mosaic virus* (WCIMV, *Potexvirus*), *Carrot red leaf virus* (CaRLV, *Luteovirus*) were not reported in Korea so far, and thus they are some examples for plant quarantine from seed materials imported from other countries. These viruses have potential seed-transmission and have been reported to injure vegetables as groundnut, carrot from aboard.

PCV showed stunting ('clump'), ringspot, and line-patterns symptoms in the plants found in West Africa (Thouvenel et al., 1976). Seed transmission was known only in groundnut (5-6%) and infected plants produced 20% of diseased seedlings (Thouvenel et al., 1988). PCV incurred yield losses up to 60% and has a wide host range that includes several weeds commonly occurring in groundnut fields in India (Ghewande and Nandagopal, 1997). Indian PCV isolates are transmitted through seed (Reddy et al., 1998). The virus has also been reported to be soil-borne and possibly transmitted by nematodes (Ghanekar, 1980).

WCIMV is widespread in broad bean and caused vein-clearing, light green mottle and sometimes small light yellow spots in pea (Forster et al., 1988). It has a fairly narrow host range, and is readily transmitted by inoculation sap, but normally not by vectors. This virus was reported to be transmitted only through seed (6%) in *Trifolium partense.*

CaRLV occurs naturally in cultivated and wild carrot, and in association with *Carrot mottle virus* (Watson et al., 1964), it causes the disease known as motley dwarf, in which the reddening, yellowing, and stunting are accentuated (Murant et al., 1995). The virus complex causes serious losses in spring-sown carrots and in carrot seed crops in Britain (Watson et al., 1964) and the USA, as well as, in Australia in the 1930s and 1940s (Stubbs, 1948). The aphid, *Cavariella aegopodii,* transmitted this virus in a persistent manner but not by inoculation with sap and not seed-transmission in carrot (Stubbs, 1948).

These viruses have no evidence of spread being reported in Korea. However, these viruses enter our country through importation from the place of origin. Imported plants were divided into planting or seedling and non-planting or seedling. In case of planting or seedling purpose, virus-infected seeds were induced through secondary transmission into domestic cultivars and economical harm and social impact in our country were examined.

Recently, the amount of imported seeds was close to

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17,518 tons and ten times the amount of others. Thus, there is a requirement to construct a reasonable detection system of imported seeds to track these viruses. But, at the moment, these viruses have not been subjected to quarantine, so far.

Therefore, the purpose of this study is to establish a rapid and exact detection technique for these viruses. For this objective, we used RT-PCR technique and this powerfully detected specific target virus from quite a few viruses into seeds. Besides, information on virus genome sequence amplified specific region of these viruses. In this study, we suggested proper total RNA extraction and RT-PCR condition for these three viruses and these conditions were applied to quarantine of three viruses.

Materials and Methods

Source of virus-infected tissues. PCV and WCIMV-infected dried tissues were obtained from DSMZ and American Type Culture (ATCC), and. CaRLV-infected tissues were kindly supplied from Bejo Seed Company in the Netherlands. These viruses were officially imported with clearance from the National Plant Quarantine Service (NPQS), and all experiments were conducted under close collaboration with NPQS staff.

Total RNA extraction. Total RNAs were extracted from PCV-, WCIMV-, and CaRLV-infected tissues. All genomic RNAs were extracted with SDS-proteinase K/phenol method as described by Ryu et al. (1995) and Rneasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol (Table 1). Each sample of freeze-dry leaf tissues was ground in extraction buffer (50 mM Tris (pH 8.0), 0.1% SDS, 10 mM EDTA) with a sterilized pestle and mortar. The crude sap preparation was added with proteinase K (10 mg/ml). The reaction mixture was extracted with PCI and ethanol precipitation. The resulting precipitates of total RNA were dissolved in RNase-free distilled water.

Oligonucleotides primers design. Degenerated or specific primers were obtained from conserved regions of PCV and WCIMV sequences from the GenBank of the National Center for Biotechnology Information (PCV: L07269, X76658, AH009203, AF239731, AF239729, AF447369, AF447399, AF447400, AF447401, NC_003668; WCIMV: NC_003820, X16636, M18920). In the case of CaRLV, a pair of degenerated primer was designed by and taken from Dr. Wang H (CEH in Oxford University). The designed primer sequences and amplified size are listed in Table 2.

Table 1. Total RNA isolation protocols for RT-PCR detection of ssRNA plant virus

Step	Proteinase K method (Ryu *et al.*, 1997)	Guanidine thiocynate method	Rneasy plant mini kit (Qiagen, USA)
1	Add 200 μl extraction buffer[a] and homogenize tissue on ice		
2	Centrifuge 1200 rpm for 1 min 4°C		Add 450 μl RLT buffer and mix
3	Add proteinase K 2 μl and mix gently	Add an equal volume of 6 M guanidinium thiocyanate and mix gently	Transfer the upper aqueous phase to tube and add 225 μl absolute ethanol, mix
4	Incubate for 30 min at 37°C	Add 5 μl RNA matrix an mix well (Bio101, La Jolla, CA)	Transfer in Rneasy mini spin column and centrifuge at 10,000 rpm for 30 sec at 4 °C
5	Add an equal volume of PCI and mix	Stand of 5 min at room temperature	Add 700 μl RW1 buffer and centrifuge at 10,000 rpm for 30 sec at 4°C
6	Centrifuge at 1200 rpm for 5 min 4°C	Centrifuge at 12000 rpm for 1 min 4°C	Add 500 μl RPE buffer and centrifuge at 10,000 rpm for 30 sec at 4°C
7	Transfer the upper aqueous phase to tube and add equal volume of CI	Add 500 μl RNA washing solution and resuspension with pipet	Add 500 μl RPE buffer and centrifuge at 10,000 rpm for 2 min at 4°C
8	Centrifuge at 12000 rpm fir 5 min at 4°C	Centrifuge at 12000 rpm for 1 min 4°C	Change tube and ad 20 μl Rnase-free water and centrifuge at 10,000 rpm for 1 min at 4°C
9	Add1/2 V 7.5M NH4OAC and 3V absolute ethanol and mix	Pellet dry and elution by DEPC-Treated water	
10	Centrifuge at 12000 rpm for 20 min 4°C	Incubate for 3 min at 50°C on water bath	
11	Add 500 μl of 70% ethanol and invert	Spin down for 1 min	
12	Centrifuge at 12000 rpm for 5 min 4°C		
13	Pellet dry and 30 μl elution		

[a]Total RNA extraction buffer containing 50 mM Tris-Cl, 10 mM EDTA, 1% SDS and 0.1% 2-merchatoethanol.

Table 2. Nucleotide sequences of oligonucleotide primers used in reverse transcription-polymerase chain reaction assays of the PCV, WClMV, and CaRLV

Primer	Sequence	Position	Amplified size
PCV uni5	5'-AGYAATAACACACTCTTCTGG-3'	3778-3798[a]	631 bp
PCV uni3	5'-ACAWCTTGTCGGYACWYCC-3'	4376-4394	
WCIMV-CP5	5'-ACCACCACAGCAACCACTCC-3'	5170-5186[b]	559 bp
WCIMV-CP3	5'-GTTGACAACGTTMGTGCCACG-3'	5708-5728	
CL1-UP	5'-TTYGTNAARGGNGARCCNCAY-3'	294-314[a]	626 bp
CL2-DN	5'-NACYTCNACYTTRAANCC-3'	902-919	

[a]Numbers indicate the corresponding position in *Peanut clump virus* (NC_003668).
[b]Numbers indicate the corresponding position in *White clover mosaic virus* (NC_003820).
[c]Numbers indicate the corresponding position in *Carrot red leaf virus* (AX404765).

Table 3. PCR reaction condition for virus-specific primers

Cycle	1		35		1	1
Time (min)	3	0.5	0.5	1	10	∞
Temp. (°C)	Predenaturation	Denaturation	Annealing	Extension	Last-extension	Store
PCV uni5/3	94	94	48	68	68	4
WCMV-CP5/3	94	94	56	68	68	4
CL1UP/ 2DN	94	94	50	68	68	4

RT-PCR detection. Total nucleic acids from virus-infected tissues were used as templates for RT-PCR. RT was performed in a reaction mixture (20 μl) containing 2.5 mM $MgCl_2$, 0.5 mM of each dNTPs, 1 μl 50 pM of reverse primer, 1 × buffer, 1 unit RNasin (Roche, USA), and 2.5 units SSTII reverse transcriptase (Invitrogen, USA) at 42°C for 60 minutes. PCR was performed in a 5 μl of the synthesized cDNA, 1 × buffer, 2.5 mM $MgCl_2$, 0.04 unit DNA pol. Mix Taq (Roche, USA), 1 μl 50 pM of each virus primers (Table 2). PCR was performed in a thermal cycler (BioRad, USA). Denaturation was executed at 94°C for 3 min before starting PCR cycling. PCR cycle consisted of 30 sec at 94°C, 30 sec at primer reasonable temperature, and 1 min at 68°C. A total of 35 cycles were performed, and cycling ended with final extension at 68°C for 10 min (Table 3). RT-PCR products were

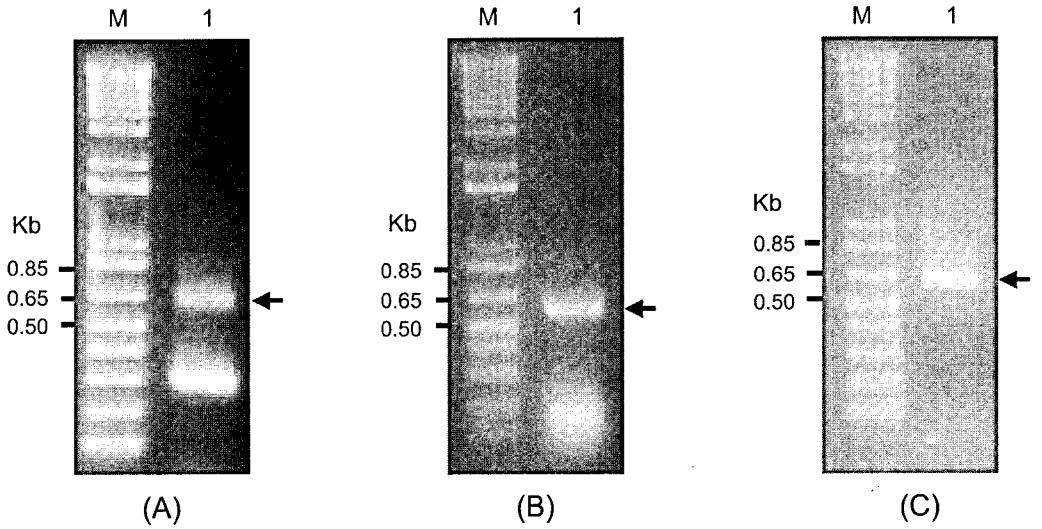

Fig. 1. RT-PCR detection of each virus using designed primer set. (**A**) Electrophoretic pattern of amplified products of conserved region of PCV-infected leaf tissues with PCV uni5/3 primer set. Lane M: 1 kb plus DNA ladder, Lane 1: PCV total RNA. (**B**) Electrophoretic pattern of amplified products of conserved region of WCMV-infected leaf tissues with WClMV-CP5/3 primer set. Lane M: 1 kb plus DNA ladder, Lane 1: WCMV total RNA (**C**) Electrophoretic pattern of amplified products of conserved region of CaRLV-infected leaf tissues with CL1UP/2DN Lane M: 1 kb plus DNA ladder, Lane 1: CaRLV total RNA.

examined by 1% agarose gel electrophoresis.

Results and Discussion

Total RNAs extraction from seed and RT-PCR analysis. Total RNAs were extracted from three methods (Table 1); and the Rneasy Mini Prep kit (Qiagen, USA) method gave the most effective and standard result. In contrast, other methods resulted in low yield and oxidization of total RNAs according to the specific character of each seed. These RNAs of seed often were not detected with RT-PCR. For valid screening test, each virus of total RNAs was isolated from variable seeds by using commercial extraction kit.

Degenerated or specific 6 primer sets designed in this

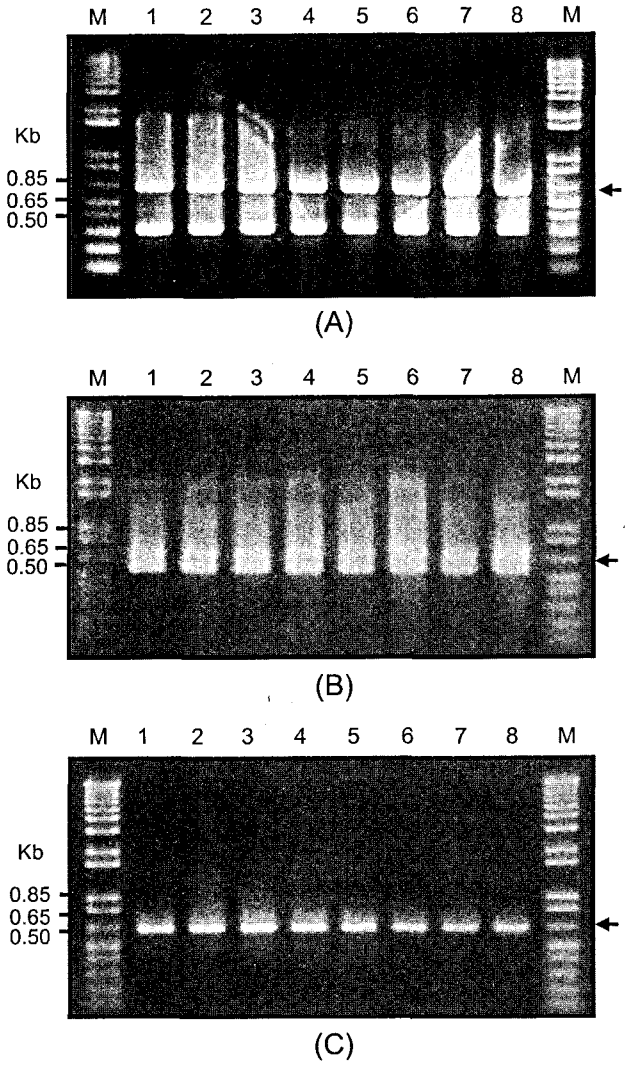

Fig. 2. Electrophoretic pattern of amplified products of changing annealing temperature of PCV-infected leaf tissues by RT-PCR (**A**). Lane M: 1 kb plus DNA ladder, Lane 1 to 8 represent annealing temperatures of 42°C, 43°C, 44°C, 46°C, 49°C, 51°C, 52°C, 53°C. (**B**) Electrophoretic patterns of amplified products of changing annealing temperature of WCMV-infected leaf tissues by RT-PCR. Lane M: 1 kb plus DNA ladder, Lane 1 to 8 represent annealing temperature of 50°C, 51°C, 52°C, 54°C, 57°C, 59°C, 61°C, 62°C. (**C**) Electrophoretic patterns of amplified products of changing annealing temperature of CtRLV-infected leaf tissues by RT-PCR. Lane M: 1 kb plus DNA ladder, Lane 1 to 8 represent annealing temperatures of 42, 43, 44, 46, 49, 51, 52, and 53°C, respectively.

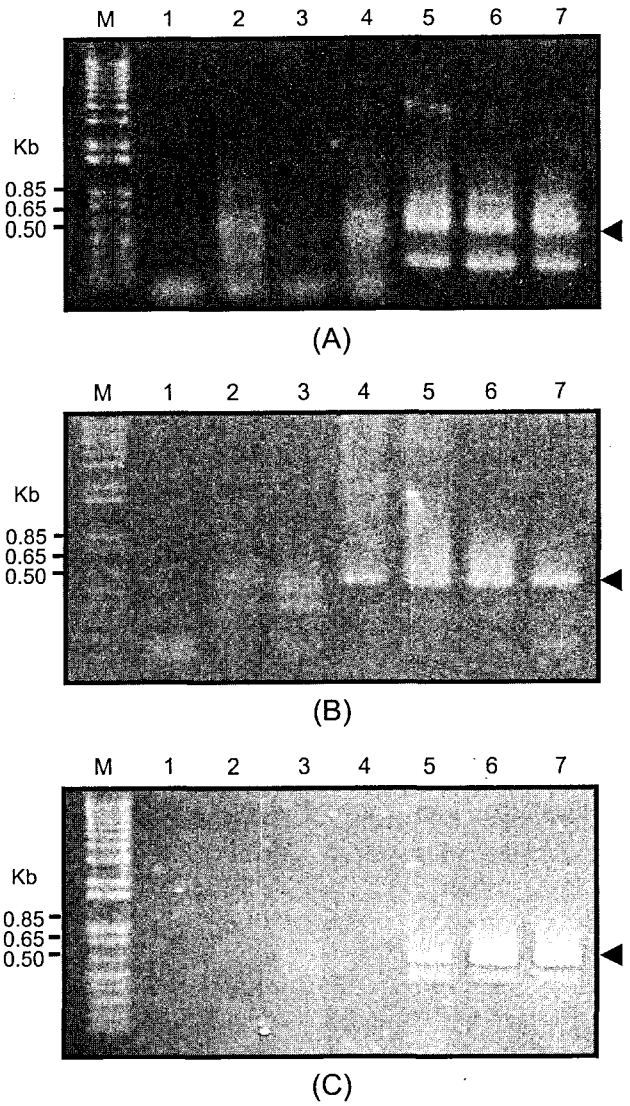

Fig. 3. RT-PCR detection by using pea seeds and PCV-infected leaves (**A**), by using pea seeds and WClMV-infected leaves (**B**). by using carrot seeds and CaRLV-infected leaves (**C**). Lane M: 1 kb plus DNA ladder, Lane 1: Total RNA of seed by Proteinase K RNA isolated method, Lane 2: Total RNA of seed by Guanidine thiocynate RNA isolated method, Lane 3; Total RNA of seed by RNeasy plant mini kit RNA isolated method, Lane 4: Total RNA of infected leaf by Proteinase K RNA isolated method, Lane 5: Total RNA of infected leaf by Guanidine thiocynate RNA isolated method, Lane 6: Total RNA of infected leaf by RNeasy kit, Lane 7: positive control.

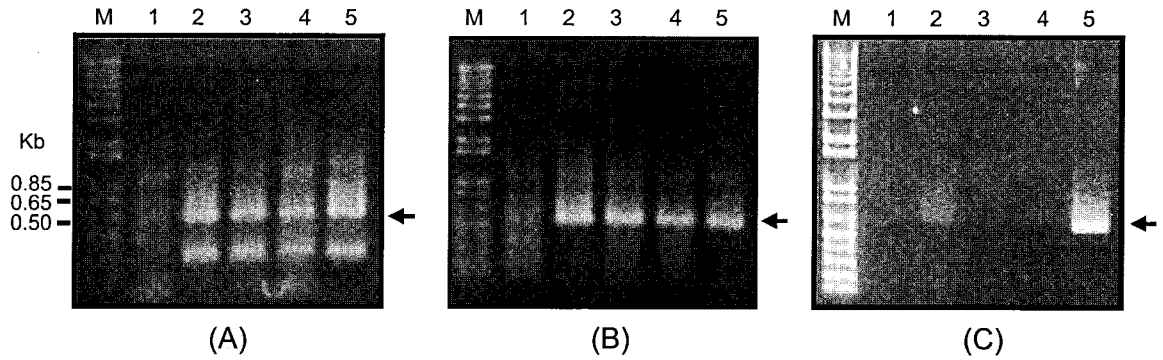

Fig. 4. Estimation of detection limit of three seed-transmissible plant viruses, PCV (**A**), WCIMV (**B**), and CaRLV (**C**) by using RT-PCR. Lane M: 1 kb plus DNA ladder, Lane1: Total RNA from seeds, Land 2; Total RNA of seeds + 1/10 diluted total RNA of virus-infected leaves, Lane 3; Total RNA of seeds + 1/100 diluted total RNA of virus-infected leaves, Lane 4; Total RNA of seeds + 1/1000 diluted total RNA of viurs-infected leaves, Lane 5; positive control.

study (Table 2) were used for detection of 3 species of seed-transmissible plant viruses. The PCR reaction was performed according to reasonable temperature of each primer sets and band of target size. A pair of PCV-uni3/5 primer detected conserved region (RNA2: ORF2e) of the genus *Pecluvirus*. A pair of WCIMV-CP3/5 primer detected conserved region (ORF5, CP) of the genus *Potexvirus*. A pair of CL1/2 primer detected conserved region (ORF1, replicase) of the genus *Luteovirus*.

PCR reaction conditions of each primer are listed in Table 3. Also, for suitable screening condition of seed transmission virus, we confirmed detection phase according to annealing temperature change of PCR reaction (Fig. 2), total RNA extraction method (Fig. 3), and 1-1/100 dilution of total RNA (Fig. 4).

PCV-uni5/3 primer set amplified PCV-specific fragments of 630 bp size (Fig. 1A). A suitable annealing temperature of this primer set was 48°C (Table 3). WCIMV-CP5/3 primer set amplified WCIMV-specific partial CP fragments of 559 bp size and 56°C (Table 3 and Fig. 1B). CL1UP and CL2DN primer sets amplified CaRLV-specific partial replicase fragments of 626 bp at 50°C (Table 3 and Fig. 1C). Uniform detection of each viruses-infected leaves were obtained when PCR reaction changed annealing temperature at 50°C ± 5°C (Fig. 2).

Total RNAs of commercial pea seeds were detected PCV by using Guanidine thiocynate and WCIMV by using Rneasy mini kit extraction. In case of commercial carrot seeds, were not obtained CaRLV by using all of them (Fig. 3C). In case of virus-infected leaves, PCV and WCIMV were amplified by using three extraction methods, but CaRLV was not amplified by using proteinase K extraction method (Fig. 2C). For reasonable result, we selected Rneasy mini kit extraction method in our other experiments.

About tests using mechanical inoculated seeds of 1, 1/10, 1/100, 1/1000 diluted total RNAs, PCV and WCIMV were detected by using diluted total RNA to 1/1000 (Fig. 4). CaRLV could be detected up to dilution of total RNA to 1/10 (Fig. 4C).

Our results clearly show that each virus was amplified to a uniform target size by RT-PCR when total RNAs were extracted by using Rneasy extraction kit. Moreover, designed primer of three pairs was confirmed to have equal yield under ±5°C of annealing temperature and exact amplification of target size. Although total RNAs of seeds were not detected by RT-PCR, this procedure was able to detect PCV, WCMV, and CaRLV from infected seed samples.

Application of molecular detection tool. Generally, seed-transmission of virus was below an average of 1%, but the second transmission by natural contagion or vectors caused extensive damage. So, importation of infected-seeds may incur economic great loss and unstable agricultural market in the country. Actually, imported seeds were mostly isolated and propagated in plant quarantine station. As a rule, ELISA is used to test for the presence of a virus in batches of seed. ELISA usually led to unstable results because of seed properties and low concentrations of virus titers. Thus, in case of seeds with a few virus sources, these viruses were detected rapidly and with exact result by RT-PCR. PCV, WCMV, and CaRLV were not reported in the country so far. But, these viruses were in danger of infecting vegetable seed imports like bean, pea, or carrot. Therefore, testing hypothesis of virus-infected seeds must be settled immediately in domestic quarantine.

This study showed a useful model test with effective detection method of seed-transmissible viruses in plant quarantine.

Acknowledgments

This study was supported by a grant from the National

Plant Quarantine Service of the Ministry of Agriculture and Forestry in Korea in 2003 to KHR. The authors thank to Dr. Wang H (CEH in Oxford University) for sequence information of CaRLV prior to publication, and to Bejo Seed Company in the Netherlands for supplying CaRLV-infected samples.

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