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RT-PCR Detection of Three Non-reported Fruit Tree Viruses Useful for Quarantine Purpose in Korea

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A simple and reliable procedure for RT-PCR detection of *Apple stem pitting virus* (ASPV), *Cherry rasp leaf virus* (CRLV), and Cherry necrotic rusty mottle virus (CNRMV) was developed. Two virus specific primer sets for each virus were found to specifically detect each virus among fourteen sets of designed oligonucleotide primers. Total RNAs extracted from healthy and from ASPV-, CRLV- and CNRMV-infected plant tissues were used to synthesize cDNA using oligo dT primer and then amplified by virus-specific primers for each virus. Each primer specifically amplified DNA fragments of 578 bp and 306 bp products for ASPV (prAS CP-C and prAS CP-N primers, respectively); 697 bp and 429 bp products for CRLV (prCR4 and prCR5-JQ3D3 primers, respectively); and 370 bp and 257 bp products for CNRMV (prCN4 and prCN6-NEG 1 primers, respectively) by RT-PCR. DNA sequencing of amplified DNA fragments confirmed the nature of each amplified DNA. Altogether, these results suggest that these virus specific primer sets can specifically amplify viral sequences in infected tissues and thus indicate that they can be used for specific detection of each virus.

Keywords **:** *Apple stem pitting virus* (ASPV), Cherry necrotic rusty mottle virus (CNRMV), *Cherry rasp leaf virus* (CRLV), quarantine, RT-PCR, virus detection

Fruit trees are large income crop for farm in the world and apple and cherry trees are one of the important fruit trees covering about 13% of total fruit tree production. Fruit tree viruses were often regarded as minor causal agents for tree diseases since viruses infecting fruit trees frequently cause few symptoms (Kim, 1999). Virus-infected cells are not generally killed but they replicate and produce progeny viruses that further infect tree. Unlike virus-infected crops and fodder that still can be used, fruit trees and ornamental crops can be totally lost or can cost a massive economic damage by adversely affecting fruit quality and/or productivity. *Apple stem pitting virus* (ASPV), *Cherry rasp leaf virus* (CRLV), and Cherry necrotic rusty mottle virus (CNRMV) are found as common pathogens in commercial apple and cherry cultivars causing significant yield losses and thus economically affecting farm income (Desvignes, 1999; James and Upton, 2002; Stouffer and Fridund, 1989). In Korea, however, a large number of economically important fruit tree viruses including ASPV, CRLV, and CNRMV are yet to be fully identified and their relationships to similar viruses characterized in other countries are still remained to be determined.

ASPV is widespread in commercial apple cultivars, which are symptomlessly infected, unless they grafted on sensitive rootstocks (Yanas et al., 1990). Symptoms include pitting of the woody cylinder, or epinasty and decline that develop in some ornamental *Malus* species and in Virgina Crab and Spy 227 indicators (Martelli and Jelkmann, 1998). CRLV is readily transmitted by grafting and by dagger nematodes (Nylnad et al., 1969). Cherry rasp leaf disease in cherry reduces fruit production, tree vigor and life expectancy (Nyland, 1976). It also infects several weeds such as dandelion. CRLV spreads in the North American region and the Pacific region including Canada, New Zealand, South Africa, and the USA. *Prunus avium* raspleaf symptoms observed in many countries were found to be associated with these symptoms in many cases (Hansen et al., 1974). CNRMV is a serious disease of sweet cherry in North America, Europe and New Zealand (Wadley and Lipman, 1976). The most important disease characteristics are brown angular necrotic spots, rusty chlorotic areas, shot holes of the leaves, blisters, gum pockets and general necrosis of the bark. The disease can be transmitted by grafting but not by mechanically (Rott and Jelkmann, 2001).

Although these three viruses are found in many other countries and spreading to the other regions every year, no evidence of spread was reported in Korea. After the liberalization of regulations for import of plants and plant materials, there is a potential risk of introducing several destructive pathogens and pests, especially viruses and viroids. Implementation of adequate diagnostic methods has become imperative to prevent the introduction of new pathogens and results in saving of millions of dollars. Unfortunately, routine diagnostic technique, which is reliable,

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inexpensive and rapid, for the detection of ASPV, CRLV, and CNRMV is not available. For this purpose, therefore, RT-PCR technique offers a powerful alternative for these fruit tree viruses. The PCR is a very powerful method that has greatly facilitated detection of plant viruses that would be difficult or time consuming to detect using conventional assays (for review, see Hadidi et al., 1995). The availability of genomic RNA sequence information has facilitated the implication of RT-PCR due to the easiness of designing oligonucleotide primers that are specific for the detection of fruit tree viruses. The RT-PCR assays have been used for the detection of several viruses infecting woody plants (Ito et al., 2002; Kokko et al., 1996; Kundu, 2003; Nolasco et al., 1993; Rosner et al., 1997; Spiegel et al., 1994; Sugieda et al., 1998; Vitushkina et al., 1994). In this study, attempts were made to develop and testify RT-PCR protocols that were simple, reliable for the routine diagnosis of ASPV, CRLV and CNRMV. RT-PCR protocols developed in this study can be employed for quarantine of these viruses.

Materials and Methods

Viruses-infected tissues. CRLV-infected tissue was obtained from American Type Culture Collection (ATCC). ASPV-infected tissues were obtained from Dr. Sano, Faculty of Agriculture and Life Science in Hirosaki University. CNRMV-infected tissues were obtained from Irrigate Agriculture Research & Extension center in Washington State University. These viruses were imported upon permission 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 healthy *Cucumis sativus* leaves and ASPV-, CRLV-, and CNRMV-infected apple and cherry tissues, respectively, using TriZol™ (Giboco BRL, USA) and S.N.A.P™ Total RNA Isolation Kit (Invitrogen, USA) according to the manufacture's protocols. Total RNAs were also extracted by resuspending grounded tissue in STE buffer (25 mM Tris-HCl, pH 7.0, 50 mM NaCl and 0.5 M EDTA) containing 2% bentonite (w/v) followed by phenol: chloroform:isoamylalcohol (25:24:1) extraction and ethanol

Table 1. Nucleotide sequences of oligonucleotide primers used in reverse transcription-polymerase chain reaction assays of the ASPV, CRLV, and CNRMV

Primer	Sequence	Tm (°C)	Length (bp)
prAS CP-N5	5'-CCW TTT GAA ACT GGC ACW GC-3'	61	578
prAS CP-N3	5'-TGC AGC ATG AGG TTC C-3'	48	
prAS CP-C5	5'-GTA CTY TGA GGC AGT ATT GTG C-3'	60	306
prAS CP-C3	5'-CAA TTT TTC CCC CAG TGA C-3'	62	
prCR1F	5'-GACCACGATCCTTATGTAGCG-3'	54	621
prCR1R	5'-CAGATGTTAACAGAAGACGAATTT-3'	54	
prCR2F	5'-GACTTAGGTAGAGATCGCTGC-3'	50	803
prCR2R	5'-CCCTCAACAAGGTCAGTAGC-3'	51	
prCR3F	5'-GCTATTTTCCCTTCGCAAG-3'	61	522
prCR3R	5'-CCTTAGAGACGTATTTACACTGTC-3'	58	
prCR4F	5'-GGAGTGGCACTGTACATATTCC-3'	62	697
prCR4R	5'-CCTGAAGTGGGTAGGTGG-3'	61	
prCR5F-JQ3D3F[a]	5'-TGCCCGACCAAATGCCAG-3'	58	429
prCR5R-JQ3D3R[a]	5'-AGGAGAAGTATTAACAACTG -3'	64	
prCR6F-JQ2C1F[a]	5'-GCCAGTTTCTCCAGTGAACC-3'	62	
prCR6R-JQ2C1R[a]	5'-CAGTTGAAACGGATTTAAAC-3'	54	370
prCN2F	5'-GRAGCACAATCTACAACCCT-3'	48	580
prCN2R	5'-CTCCAATAGTGMAGCTCATC-3'	48	
prCN3F	5'-GTGATTAGAGCTGTTAGTGA-3'	41	716
prCN3R	5'-TTYAGKGCCACCTCAAAAGT-3'	52	
prCN4F	5'-AGCACWRTTAGGAGCTACTG-3'	50	370
prCN4R	5'-TATCATTCATCACCACCAAT-3'	47	
prCN5F-NRM48U[b]	5'-TTAATGATCTTCGTGGCTTGTTG-3'	56	–
prCN5R-NRM48L[b]	5'-GAATTGACTCCTCGGTGGGTTTA-3'	58	
prCN6F-NEG1U[b]	5'-AGTTCGCAGCYTTTGAYTTYTTTG-3'	60	257
prCN6R-NEG1L[b]	5'-GAKTGGRWTTGCAGRGGTTTATCA-3'	64	
prCN7F-CGRMVU[b]	5'-GCAGCCTTTGACTTTTTTGAG-3'	53	366
prCN7R-CGRMVL[b]	5'-CCTATAGCCAGTCTTCATATTATG-3'	49	

[a] Oligonucleotide primers used for the detection of CRLV were adapted from James et al. (1999).

[b] Nucleotide sequences of these primers used for the detection of CNRMV were adapted from the information given in the web site at http://www.boku.ac.at/iam/pbiotech/phytopath/d_slr.html.

precipitation. Integrity and relative concentration of purified transcripts were analyzed by agarose gel electrophoresis at 4°C.

Oligonucleotide primer design. Viral sequence or conserved regions were obtained from sequence alignments of all available sequences from the GenBank of the National Center for Biotechnology Information for each virus (ASPV: AF491930, AF491931, AF438521, AF438522, AF495382, AF345895, AF49194, AF491929, AF345893, AF345892; CRLV: AY122330; CNRMV: AF237816, CNE291761, CNE297268) and additionally primers were prepared from designed primer information given in the literature and web site (CRLV: James et al., 2001); CNRMV: http://www.boku.ac.at/iam/pbiotech/phytopath/d_slr.html). The designed primer sequences, Tm values and expected amplified fragment sizes of each primer are listed in Table 1.

RT-PCR. Approximately 1.5 μg of total RNA (5 μL) and 75 pmol (3 μL) of the oligo dT primer (18mer) were denatured by heating at 99°C for 5 min. Reverse transcription (RT) was carried out in a final 20 μL volume obtained by adding 12 μL of a RT reaction mix (2 μL 10×RT buffer, 4 μL 2.5 mM dNTPs mix, 1 μL Mu-MLV Reverse transcriptase (NEB), and made up to volume with DEPC-dH_2O). RT was carried out at 4°C for 60 min. When RT was completed, 50 μL of a PCR reaction mix (3 μL 2.5 mM dNTP mix, 5 μL 10×PCR buffer, 1 μL virus-specific primers (25 pmol each), and 0.5 μL Ex Taq DNA polymerase (TaKaRa, Japan)) was added. Amplification was carried out for 34 cycles with gradient cycling profile for each primer set and the optimum temperatures for each primer set for PCR reaction were determined.

Cloning and sequencing. The PCR product was purified and cloned into pGEM-T EASY vector (Promega, USA). Inserts were sequenced by the dideoxynucleotide chain termination method as previously described (Jung et al., 2002; Kim et al., 2003) using the ABI prism™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prime 3700 Genetic Analyzer (Perkin Elmer, USA) located at the NICEM (SNU). The sequence and deduced amino acid sequences were compiled, analyzed and compared using the LaserGene program (DNASTAR, USA), the ClustalW method with weighted residue weight table, the PAUP software (Sinauer Associates, USA) and the BLAST program (Altschul et al., 1997). The sequences of previously reported viruses were retrieved from the GenBank database and used for analyses.

Results and Discussion

RT-PCR analysis. Total RNAs extracted by three different

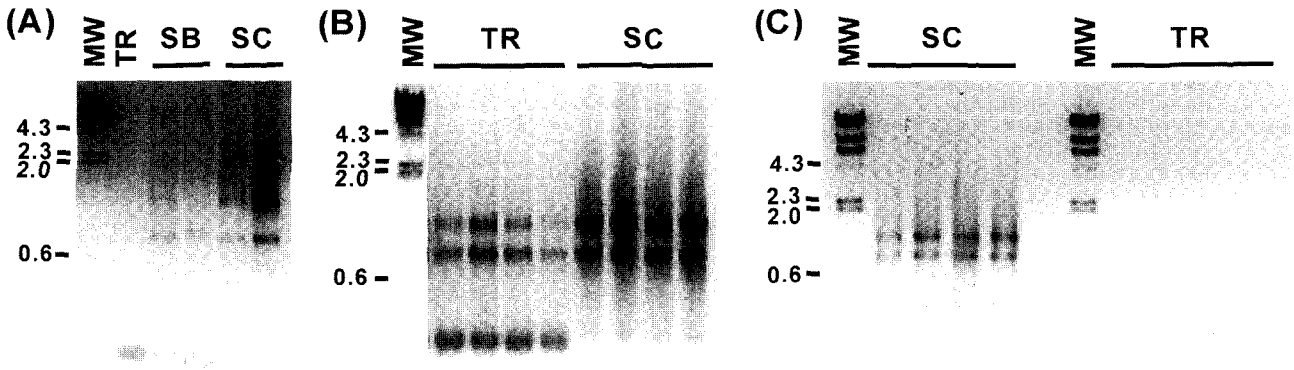

Fig. 1. Total RNAs extracted from ASPV (**A**), CRLV (**B**), and CNRMV (**C**) infected tissues with the extraction procedure indicated and separated on 1% agarose gel. Total RNAs were extracted by using Trizol reagent (TR), STE buffer containing bentonite (SB), or RNA extraction kit employing silica column (SC). Lane MW indicates molecular size markers, λ DNA digested with *Hin*dIII. Numbers on the left of each gel indicate sizes of DNA digested with *Hin*dIII in kilobases.

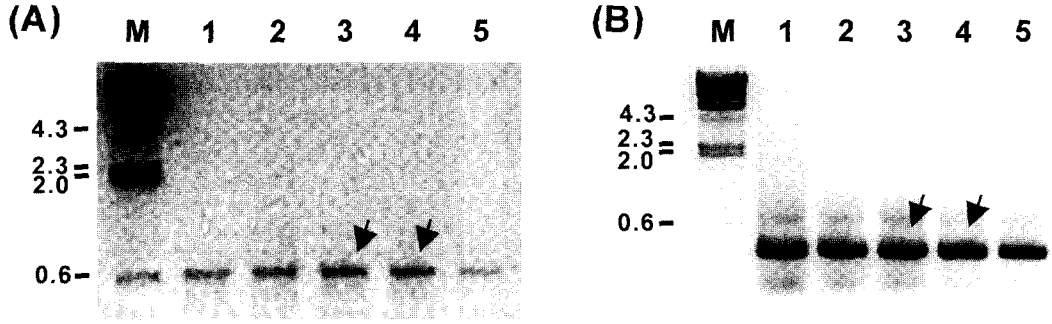

Fig. 2. Comparison amplified DNA fragments after gradient PCR. Total RNAs extracted from ASPV-infected leaves and prAS CP-N (**A**) and prAS CP-C (**B**) primer sets were sued. Lanes 1 to 5 represent annealing temperatures of 52, 55, 57, 59, and 61°C in panel A and 45, 48, 52, 57, and 61°C in panel B. Lane M indicates molecular size markers, λ DNA digested with *Hin*dIII. Amplified DNAs were separated on 1% agarose gel and visualized by ethidium bromide staining.

Table 2. PCR reaction conditions for virus-specific primers

Cycle	1			34			1	1
Time (min)	5	10	10	40 s	1	3	15	∞
Temp. (°C) [a]	A	B	C	A	B	C	C	Store
prAS CP-N	94	**49**	72	94	**57**	72	72	4
prAS CP-C	94	**49**	72	94	**57**	72	72	4
prCR4	94	**45**	72	94	**50**	72	72	4
prCR5-JQ3D3	94	**45**	72	94	**62**	72	72	4
prCN4	94	**45**	72	94	**50**	72	72	4
prCN6-NEG1	94	**45**	72	94	**62**	72	72	4

[a] A to C indicate temperatures used for denaturation, annealing, and extension for PCR reaction.

extraction methods as described above. Of these extraction conditions tested, the method employing silica column (Invitrogen) gave the most reproducible results. RNA bands extracted using Trizol reagents or the conventional STE buffer were sometimes not clear or lower intensity (Fig. 1). In contrast, total RNAs extracted by employing commercial kits resulted in high RNA yields regardless of tissue samples used. The purity and the low yield of total RNAs may be a handicap for the detection of fruit tree viruses in many cases. These RNAs also produced reliable results when used for RT-PCR compared to the other RNAs (data not shown). Therefore, for the screening of oligonucleotide primers specific for the detection of each virus total RNAs prepared by using commercially available kit were used.

A total of 14 primer sets were designed and tested for the specific detection of each virus. To obtain suitable annealing temperature, gradient PCR reaction was conducted for each primer set (Fig. 2). Although we designed and tested 14 primer sets, we obtained expected results from two sets of primers for each virus including prAS CP-N and prAS CP-C primer set for ASPV detection, prCR4 and prCR5-JQ3D3 primer set for CRLV detection, and prCN4 and prCN6-NEG1 primer set for CNRMV detection. The other primer sets were not effective for the detection of each virus. PCR reaction conditions for each selected primer set are listed in Table 2.

prAS CP-N and prAS CP-C primer set amplified ASPV-specific fragments of 578 bp and 306 bp (Fig. 3A) while prCR4 and prCR5-JQ3D3 primer set produced CRLV-specific fragments of 697 bp and 429 bp (Fig. 3B). prAS CP-C primer set was more effective than prAS CP-N primer set for the detection of ASPV (Fig. 3A, lanes 1 and 2, respectively). Selected primers prCN4 and prCN6-NEG 1 set produced 370 bp and 257 bp (Fig. 3C). In woody hosts, reliable detection was obtained when using either infected leaves or dormant budwoods. Multiple or non-specific

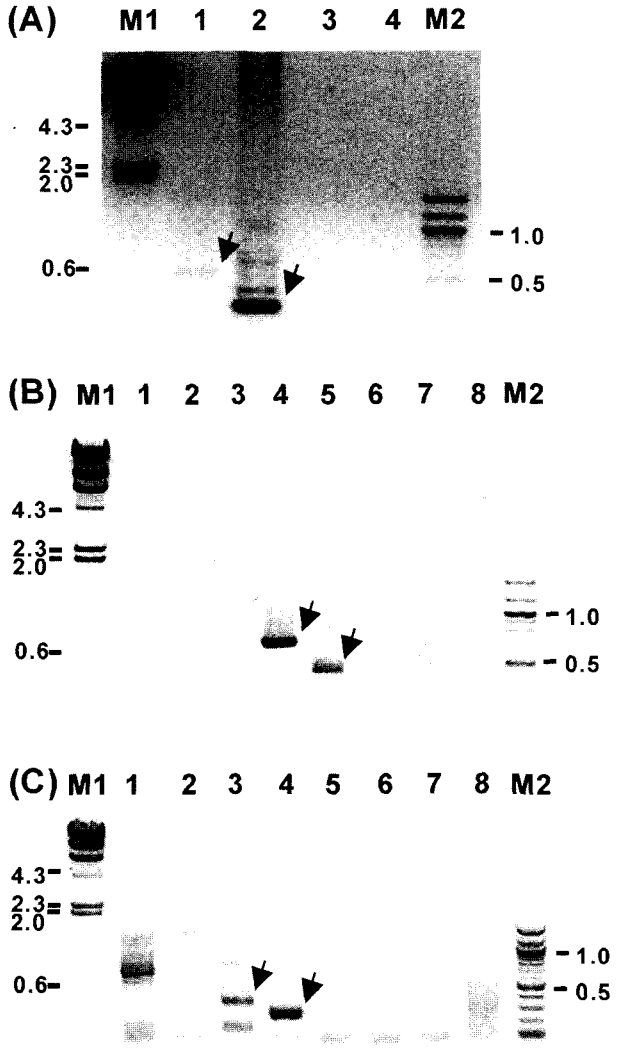

Fig. 3. RT-PCR detection of each virus using designed primer set. (**A**) ASPV detection. cDNAs were synthesized using total RNAs from ASPV- (lanes 1 & 2), CRLV- (lane 3), and CNRMV (lane 4) infected tissues. PCR amplified fragment(s) using prAS CP-N (lane 1) and prAS CP-C (lanes 2 to 4) are separated on a 1% agarose gel. (**B & C**) CRLV and CNRMV detection, respectively. Lanes 1 to 6 in panels B and C represent PCR amplified product(s) using primer sets prCR 1-6 and prCN 2-7, respectively. Lanes 7 and 8 represent (–) control amplification for prCR 4 and prCR 5-JQ3D3 primer sets using cDNA synthesized with CNRMV-infected tissues and for prCN 4 and prCN 6-NEG1 primer sets using cDNA synthesized with CRLV-infected tissues, respectively. Arrows indicate expected fragments amplified by each PCR. Lanes M1 and M2 indicate molecular size markers, DNA digested with *Hin*dIII and 1 kb ladder (New England Biolabs, Beverly, MA), respectively. Numbers on the left of each gel indicate sizes of DNA digested with *Hin*dIII in kilobases.

bands were observed when primer sets prCN2 and prCN3 were used for the detection of CNRMV (Fig. 3C, lanes 1 and 2). The total RNA extracted from all healthy controls or from the other virus-infected woody tissues gave negative

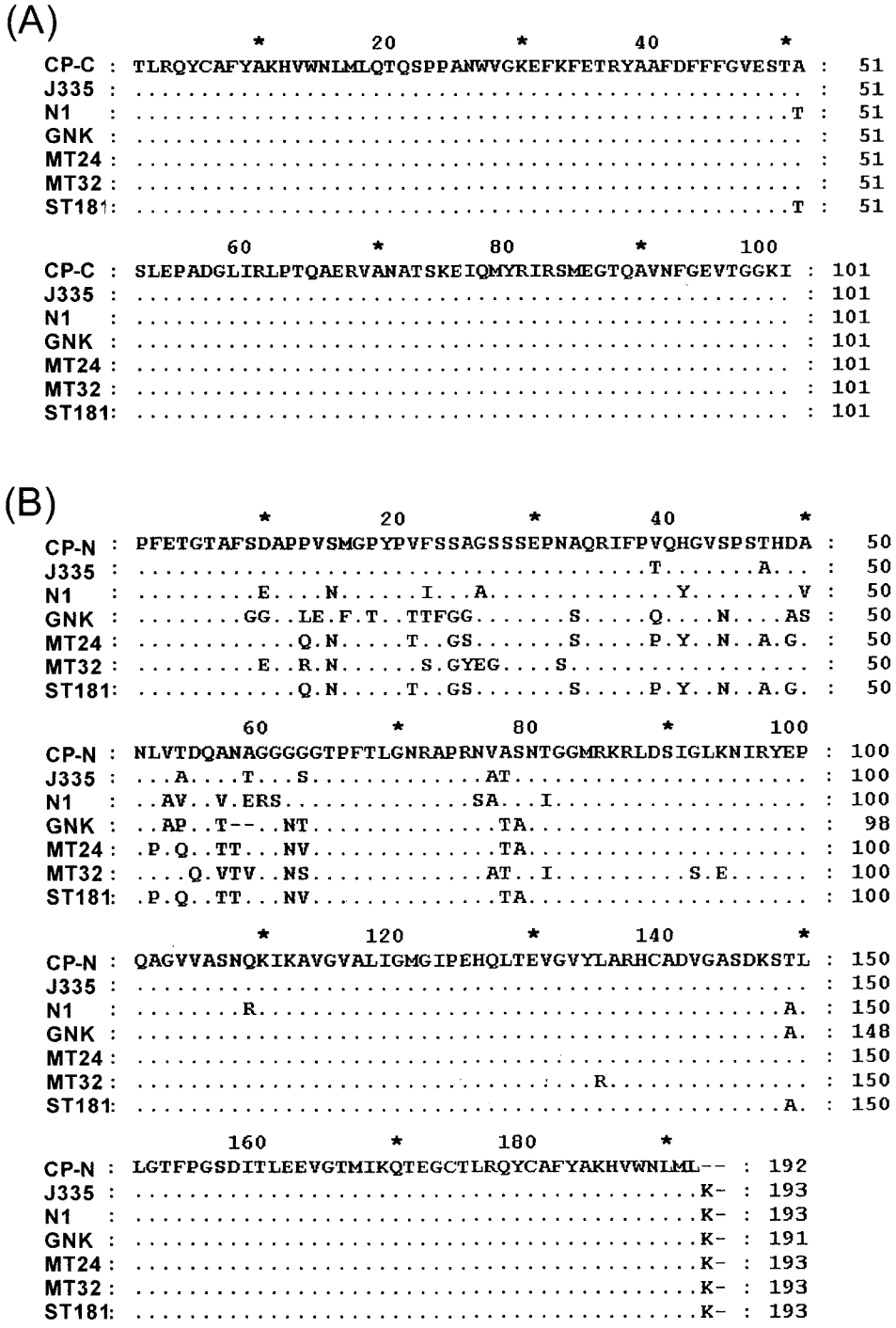

Fig. 4. Alignment of the putative amino acid sequences for prAS CP-C (**A**) and prAS CP-N (**B**) amplified sequence with previously reported ASPV sequences. Shown are sequences for ASPV isolates J335 (AF491930), N1 AF491931), GNK III (AF491929), MT24 (AF438522), MT32 (AF438521), and ST181 (AF495382). Asterisks denote the tenth position from the previously numbered amino acids.

results (Fig. 3A, lanes 3 and 4; B & C, lanes 7 and 8).

Although the leaves were more suitable source for total RNA extraction and subsequent virus detection by RT-PCR, homogenization of the other wood tissues followed by RNA extraction through silica column and RT-PCR allowed specific detection of the target virus consistently. This procedure described in this study is relatively simple and can be easily applied to many other viruses infecting wood

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                *         20         *         40         *         60
prCR4 : SGTXHIPFSSSPGSYTKNKHPKLLFVFPGGISGPSGETIHVNIQVRDILNFSGLGHQLLK :  60
CRLV  : ...V.................................................... :  60

                *         80         *         100        *         120
prCR4 : PILAAEGPDPFSFHLFYLHCGTLKTESLNKGGMWCVPVSPVNLAAMKHGAGSSLVFNESF : 120
CRLV  : ..............C.................................T.......... : 120

                *         140        *         160        *         180
prCR4 : VSKTHNWLHYMASCTAYWRGTLTYELRVTYNSRVNAVANLVAFYTSQVEDLFGFSDXPVG : 180
CRLV  : ..................................................K... : 180

                *         200        *         220        *
prCR4 : DTGIASICGDAFSVRISIPFVTPTLWLRTYRNAYDVFTSCNGSLYFHLPTS : 231
CRLV  : ..................................................... : 231
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Fig. 5. Alignment of the putative amino acid sequences for prCR4 and CRLV sequence (AY122330).

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                          *         20         *         40         *         60
prCN4          : STVRSYCSLRSFCSKYAPVIWNYGIQNNIPPANWQRRKVVEGAKFAAFDFFEAVTSAAALQ :  61
AP23816        : ..........................A.S.................................. :  61
CNE291761      : ..........................S.....................................E.... :  61
CNE297268      : ..........................S.....................................E.... :  61
CN002468       : ..........................A.S.................................. :  61
CGRMV BC       : ..........................S.....................................E.... :  61
CGRMV F        : ..........................S.....................................E.... :  61
CGRMV Ita1     : ..........................S.....................................E.... :  61
CGRMVLeb       : ..........................S.....................................E.... :  61
CGRMV N        : ..........................S.....................................E.... :  61
CGRMV P1C1     : ..........................S.....................................E.... :  61
CGRMV Wp1      : ..........................S.....................................E.... :  61
SourCGRMV      : ..........................S.....................................E.... :  61

                          *         80         *         100        *         120
prCN4          : PVEGLVRNPTDKEMTAGASLKEISLMRDEIRRGTSSTLMTEVTGGRTGLIQPIKKIGGDE : 121
AP23816        : ..A..........I.....................................Q.......... : 121
CNE291761      : .....................................................Q.......... : 121
CNE297268      : .....................................................Q.......... : 121
CN002468       : ..A..........I.....................................Q.......... : 121
CGRMV BC       : .....................................................Q.......... : 121
CGRMV F        : .....................................................Q.......... : 121
CGRMV Ita1     : .....................................................Q.......... : 121
CGRMVLeb       : .....................................................Q.......... : 121
CGRMV N        : .....................................................Q.......... : 121
CGRMV P1C1     : .....................................................Q.......... : 121
CGRMV Wp1      : .....................................................Q.......... : 121
SourCGRMV      : .....................................................Q.......... : 121
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Fig. 6. Alignment of the putative amino acid sequences for prCN4 with previously reported CNRMV and CGRMV sequences. Previously reported sequences of CNRMV isolates AP23816, CNE291761, CNE297268, and CN002468 and CGRMV isolates BC (AF33152), F (AF533150), ItA1 (AF533158), Leb (AF533157), N (AF33151), P1C1 24 (CNE291761), Wp1 (AF533155), and sour (AF017780) are used for alignment. Asterisks denote the tenth position from the previously numbered amino acids.

trees. In addition, this procedure is also applicable to small disc of leaf, stem and bud.

Sequencing analysis. The amplified fragments by RT-PCR were cloned, sequenced and compared using the BLAST programs (Altschul et al., 1997). Database comparisons of the translated 578 bp product (prAS CP-N) and 306 bp (prAS CP-C) product produced matches with N terminal and C terminal coat protein (CP) of ASPV (Fig. 4). The

CRLV-specific primers (prCR4 and prCR5-JQ3D3), which amplified a 697 bp (prCR4) and 429 bp (prCR5-JQ3D3) fragments, corresponding to a C-terminal region of CRLV RNA-2 sequence (Fig. 5). The CNRMV-specific primers (prCN4 and prCN6-NEG1) amplified 370 bp and 257 bp fragments, respectively. These fragments were aligned with a CP region of CNRMV (Fig. 6). These were matched with a CP region of *Cherry green ring mottle virus*, which is a closely related virus to CNRMV and an additional member of *Foveavirus* (Fig. 6). Although we did not completely sequenced whole genomes of CRLV and CNRMV strains, the primer sets selected for the detection of each virus clearly showed that they could be useful for the specific detection of each virus.

Implication in fruit tree virus quarantine. Fruit tree viruses were often regarded as minor causal agents for cells and not generally killed but the viruses replicate and produce progeny viruses that further infect trees. The subtle nature of symptoms and dissemination through grafting and propagation in the past led to an accumulation of viruses in fruit trees and resulted in contaminating many cultivars (Cropley, 1968). Detection of fruit tree viruses has been limited to field indexing on some indicator trees. Field indexing on woody indicator is time consuming and often unreliable (Stouffer and Fridund, 1989). Woody plant extract contain very low concentration of virus in many cases. Therefore, application of RT-PCR for the detection of plant RNA viruses gave many advantages including high sensitivity, high specificity, and high sample throughput. It has been reported that using PCR-based assay one can claims the detection of around 10 femtograms (fg) of viral RNA (Romaine and Schlagnhaufer, 1995).

Application of RT-PCR in practical screening of plant viruses, however, is considerably limited by production of secondary metabolic products that degrade viral RNA and/or prevent RT enzyme reaction in sample extracts especially for many fruit tree varieties including *Malus*, *Prunus*, and *Pyrus* origin (Korschineck et al., 1991). Recently, it has been shown that the sodium sulphite based extraction protocol effectively inhibits polyphenolics and thus increase efficiency of RT-PCR (Singh et al., 2002). Methods using spin column employed in this study showed that it enables a rapid and efficient RNA extractions and eliminate the use of hazardous chemicals. This type of extraction procedure, thus, can be used to extract high quality RNAs and subsequent virus detection using RT-PCR. The detection of ASPV, CRLV, and CNRMV in infected leaves, stem, and bud tissue should allow screening and certification of wood stocks throughout the year and thus the technique described in this study may be implicated for the detection of other fruit tree viruses and for routine screening of these viruses for quarantine purpose.

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