

# Development of a Multiplex RT-PCR for the Simultaneous Detection of Three Viruses in Cherry Plants

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A multiplex RT-PCR (mRT-PCR) assay was developed for the detection of the recently reported viruses, *Cherry virus A* (CVA), *Little cherry virus 1* (LChV-1), and *Little cherry virus 2* (LChV-2), in cherry plants in Korea. Eight sets of primers were designed for each virus and their specificity was tested by using various combinations of mixed primer sets. From the designed primer sets, one combination was selected and further evaluated to estimate the optimum temperature and detection limits of the mRT-PCR. A newly developed mRT-PCR assay was also tested using 20 cherry samples collected in the field. This mRT-PCR assay may be a useful tool for field surveys of diseases and the rapid detection of these three viruses in cherry plants.

**Keywords:** *Cherry virus A*, *Little cherry virus 1*, *Little cherry virus 2*, Multiplex RT-PCR

As climate change and free trade agreements increase, the domestic introduction of various tropical plants into Korea is increasing. The introduction of new plants increases the potential for novel and unreported pathogens, and could have effects on the domestic ecosystem (Lee et al., 2011).

With the rising demand of cherry, one of the various subtropical crops that is considered a minor fruit crop in Korea, its cultivation area continues to increase through regional specialization (Cho et al., 2018; Kang et al., 2007). Cherry plants are categorized into two species: sweet cherry (*Prunus avium* L.) and sour cherry (*Prunus cerasus* L.); sweet cherry is more widely cultivated than sour cherry (*Prunus cerasus* L.) in Korea. Until now, seven viruses [*Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Cherry virus A* (CVA), *Cherry green ring mottle virus* (CGRMV), *Little cherry virus 1* (LChV-1), and *Little cherry*

*virus 2* (LChV-2)] from sweet cherry have been reported in Korea (Cho et al., 2013, 2014, 2018; Lee et al., 2014a). Of these, three viruses (CNRMV, LChV-1, and LChV-2) were designated as quarantine viruses by the Animal and Plant Quarantine Agency (QIA). A duplex-PCR assay has been previously developed for the detection of CGRMV and CNRMV (Lee et al., 2014b). However, simultaneous diagnostic methods, which include LChV-1 and LChV-2, have not been developed in Korea. In recent years, CVA and LChV-1 have been reported in *Prunus* species such as peach tree (*Prunus persica*) and Japanese apricot (*Prunus mume*) (Jo et al., 2017; Lim et al., 2015; Lim et al., 2017, Koinuma et al., 2016). Moreover, LChV-2 is known to be transmitted by the apple mealybug (*Phenacoccus aceris*) (Signoret) and grape mealybug (*Pseudococcus maritimus*) (Ehrhorn) (Mekuria et al., 2013; Raine et al., 1986). To minimize the damage caused by these viruses, a rapid and accurate diagnostic system is vital. mRT-PCR has the benefit of being able to detect multiple target pathogens in a single reaction, reducing the time and cost of diagnosing these samples individually (Asano et al., 2015; Yao et al.,

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2014). This study was, therefore, undertaken to develop an mRT-PCR assay for the simultaneous detection of CVA, LChV-1, and LChV-2 in cherry plants.

**Plant materials.** Two cherry leaf samples were put together and then grind the samples in liquid nitrogen for a positive control. One sample was infected with CVA and LChV-2, and the other sample was infected with CVA and LChV-1. These samples were registered in NCBI GenBank with accession no. MF083705 (LChV-1) and MF083705 (LChV-2) (Cho et al., 2018).

**Total RNA extraction and cDNA synthesis.** Total RNA was extracted from the samples using the Nuclisens Easy-Mag bio-robot (Biomérieux, Marcy l'Etoile, France) and eluted in a volume of 50 µl. The concentration and purity of the extracted total RNA was measured using Tecan Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland) and was estimated to be 88.32 ng/µl. First-strand cDNA was synthesized using M-MLV (Invitrogen, Life Technologies, Carlsbad, CA) with random N25 primers, according to the manufacturer's instructions.

**Virus-specific primer design.** To design each virus-specific primer pair, the complete nucleotide sequence of each virus was downloaded from the NCBI GenBank database. Six types of CVA isolates and one other member of the *Capillovirus* genus were used for CVA-specific primer design. The LChV-1 and LChV-2-specific primer pairs were designed similarly to the CVA primer. All downloaded sequences were aligned using DNAMAN software ver. 7.0 (Lynnon BioSoft, Quebec, Canada). Specific primer pairs were designed based on highly conserved regions of their target viruses, with a total of eight primer pairs de-

signed for each virus.

**PCR amplification and primer selection.** The specificities of the designed primers were tested by monoplex RT-PCR with 2X TOPsimple DyeMix (aliquot)-HOT premix (Enzynomics, Daejeon, Korea). For PCR amplification, 3 µl of the cDNA template was used in a 20 µl reaction volume. PCR conditions were as follows: initial denaturation at 95°C for 10 min; 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 5 min. The sizes of the PCR products were confirmed on a 1% agarose gel stained with EcoDye DNA Stain (SolGent Co., Daejeon, Korea). Amplicons were purified using the Expin PCR SV kit (GeneAll, Seoul, Korea) and sequenced directly using each primer. All species-specific primers designed for the three viruses were successfully amplified by the monoplex RT-PCR reaction and direct sequencing results confirmed the target viral sequences. Of three target viruses, four CVA primer pairs were selected, based on band intensity, and then combined with LChV-2 primer pairs for duplex RT-PCR. Among the 22 duplex RT-PCR (CVA+LChV-2) combinations, seven were selected, and then 28 primer pairs were combined with LChV-1 for mRT-PCR. Ten primer combinations were found to be effective in mRT-PCR, and finally one combination [CVA (270 bp)+LChV-2 (570 bp)+LChV-1 (427 bp)] was selected based on band intensity, sharpness, and target sizes (Table 1, Fig. 1A).

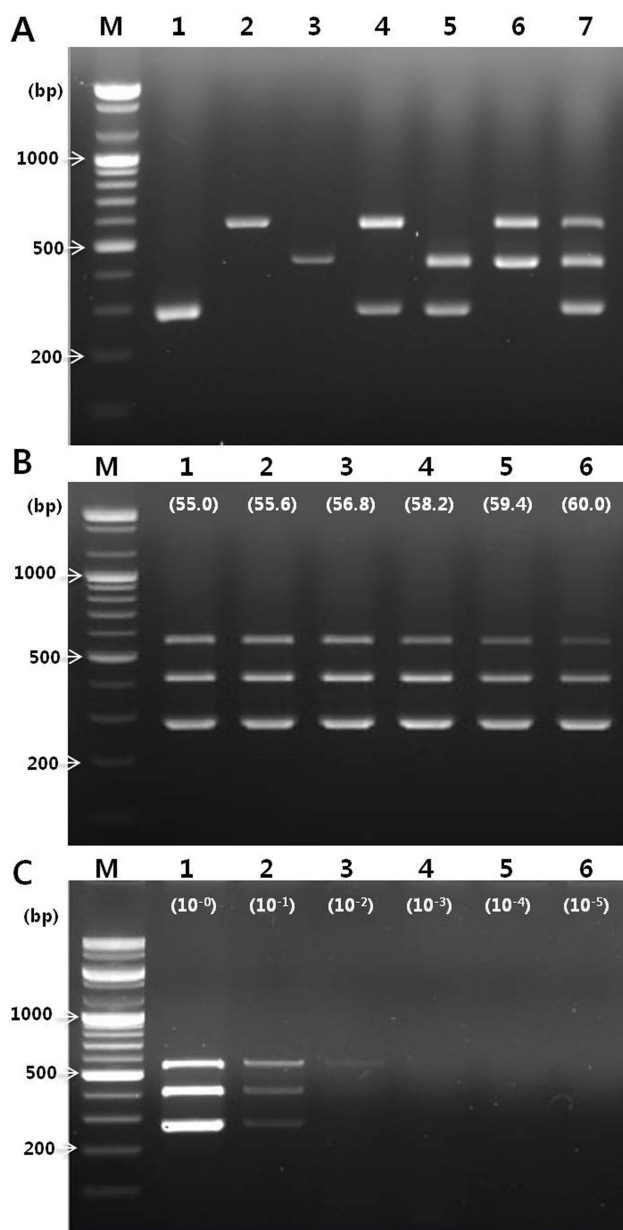
**Optimization and sensitivity of multiplex-PCR.** Different concentrations of primer pairs and annealing temperatures were tested to optimize the simultaneous detection of the three viruses. A range of annealing temperatures were compared, from 55 to 60 °C, using PCR gradient analysis.

When mRT-PCR was performed using the same primer

**Table 1.** Oligonucleotide sequences of a multiplex-PCR for the detection of the three viruses

Virus name	Primer name	Sequences (5' to 3')	Target*	Amplicon size
CVA	CVA-D-F2	AGTGCTCACAGCTGTTAAAAG	MP	270 bp
	CVA-D-R3	CTAAAACCTGCTTGGCATGC		
LChV-2	LCh2-D-F1	TCGTGTATAGACCTTTGTATGA	p53	570 bp
	LCh2-D-R1	AAACTTCGAGAACCGGACTC		
LChV-1	LCh1-D-F2	GAAGCCTTGAACTTCTGACA	CP	427 bp
	LCh1-D-R3	CTAATTTAGTTGTAGTCGATTGT		

\*All primers for each virus were designed within the conserved regions: CP (coat protein), MP (movement protein), p53 (protein 53).



**Fig. 1.** (A) Simultaneous detection using the selected primer sets for the three viruses (*Cherry virus A*, CVA; *Little cherry virus 1*, LChV-1; and *Little cherry virus 2*, LChV-2) by multiplex PCR. Lane M: 1kb DNA ladder (Bioneer, Daejeon, Korea), Lane 1: *Cherry virus A* (CVA), Lane 2: *Little cherry virus 2* (LChV-2), Lane 3: *Little cherry virus 1* (LChV-1), Lane 4: CVA+LChV-2, Lane 5: CVA+LChV-1, Lane 6: LChV-2+LChV-1, Lane 7: CVA+LChV-2+LChV-1, (B) Optimization temperature test, (C) Sensitivity test.

concentrations, the resulting CVA positive band intensity was strong, while LChV-1 and LChV-2 were weak. Therefore, mRT-PCR was performed by adjusting the primer concentration to 5, 10, and 20 pmole. As a result, the positive band intensity of the three viruses was equaled. Among the five

different annealing temperature conditions, the optimal annealing temperature was confirmed as 56.8 °C (Fig. 1B).

To confirm the sensitivity of the PCR detection assay, a 10-fold serial dilution was conducted up to  $10^{-5}$  by monoplex RT- and multiplex RT-PCR, using cDNA templates. In the monoplex RT-PCR, the detection limit was  $10^{-2}$  for CVA and  $10^{-1}$  for both LChV-1 and LChV-2. Using mRT-PCR, amplification products were observed up to  $10^{-1}$ , whereas only CVA was detected up to  $10^{-2}$  (Fig. 1C). The annealing temperature and individual primer concentrations are suitable for detecting all three viruses. These results show that the detection limit is lower than the total amount of RNA which was extracted from other crops and/or plants (Lee et al., 2017; Nam et al., 2015; Yu et al., 2013). In our study, we believe the low detection limit was due to the low concentration of the first extracted total RNA and that the detection limit range could be increased if higher concentration of total RNA was extracted.

**Application for cherry plants infected in field.** In May 2018, a total twenty cherry leaf samples were collected from Gyeongju ( $n$ : 12) and Daegu city ( $n$ : 8) for virus testing. The collected samples showed typical virus-like symptoms, including mosaic, malformation, and red ring spots on the leaves (Fig. 2). All samples were tested using the developed monoplex RT- and mRT-PCR. The test results showed that all twenty samples were positive for CVA, four were coinfecting with CVA+LChV-1, and six were coinfecting with CVA+LChV-2. Monoplex RT-PCR results were consistent with the mRT-PCR assay (Data not shown). The monoplex RT- and mRT-PCR results did not show any non-target bands or PCR dimers. These results imply that the newly developed mRT-PCR assay for the three viruses (CVA, LChV-1, and LChV-2) enable their simultaneous detection in infected cherry plants.

As previously reported by Cho et al. (2014), the CVA and CNRMV co-infection rate was very low (0.6%) in sweet cherry samples. To confirm this in our samples, an additional PCR was carried out to detect CNRMV using same primer pairs. However, our survey results confirmed that the CVA and CNRMV infection rate (50%) was higher than the above results. Cherry virus surveys have only been performed in certain areas, and a nationwide survey will therefore be needed to assess the prevalence of cherry virus diseases in Korea. The mRT-PCR we have developed and the overall findings of this study could contribute to the development of new methods



**Fig. 2.** Virus symptoms observed on the leaves of cherry plants. (A) Malformation symptoms and coinfection with CVA and LChV-2, (B) Vein clearing and coinfection with CVA and LChV-1, (C) Mosaic and infection with CVA.

for the rapid detection of plant viruses and field surveys.

### Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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