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Development of Diagnostic PCR System for Three Seed-transmitted Quarantine Viruses Associated with *Cucurbitaceae*

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The *Cucurbitaceae* are a plant family that consist of over a hundred genera, the most important of which are squash, pumpkin, zucchini, some gourds, cucumber and watermelon. These are among the top imported seeds in Korea. At the time of their import, the *Squash mosaic virus* (SqMV), the *Cucumber green mottle mosaic virus* (CGMMV) and the *Kyuri green mottle mosaic virus* (KGMMV) are designated as regulated viruses for quarantine in Korea. This study was conducted to develop specific primer sets for easy and rapid detection of SqMV, CGMMV and KGMMV. RT-PCR with the nested PCR primer sets and modified positive control plasmids were capable of highly sensitive detection and verification of such viruses. In addition, developed diagnostic PCR systems applied to quarantine sites detected 47 cases of SqMV, 67 cases of CGMMV and 17 cases of KGMMV between 2010 and the first half of 2014.

Keywords: *Cucurbitaceae*, Plant quarantine, SqMV, CGMMV, KGMMV

Due to import liberalization, the world seed market has continuously expanded [17]. In Korea, more than about 6,000 cases (about 3.1 tons per case) of seeds have been imported annually [5]. Seeds can be imported to Korea via air, ship, mail and carry-on baggage (www.qia.go.kr) with the accompanying risk of introducing seed-transmitted diseases and insect pests. Accordingly, inspection is carried out by designating different seed specific quarantine pathogens in Korea [18]. Among the seed-transmitted pathogens, the biggest potential problems are viruses. Total of 32 seed-transmitted viruses are designated as quarantine pathogens, and relevant inspection is routinely carried out [1, 5]. According to the Pest Information System of the Animal and Plant Quarantine Agency, the annual average imports of *Cucurbitaceae* crops (e.g., watermelon, pumpkin, gourd, oriental melon and melon) in the last 3 years (2010-2012) were 1,220 cases that accounted for >20% of the imported seeds in that period. As for the quarantine inspection of *Cucurbitaceae* seed-transmitted viruses, the *Melon necrotic spot virus* was reclassified as non-quarantine based on the amendment of the Korea Risk Assessment in 2012. Viruses that are designated as the targets of *Cucurbitaceae*-seed-transmitted quarantine in Korea include *Squash mosaic virus* (SqMV), *Cucumber green mottle mosaic virus* (CGMMV) and *Kyuri green mottle mosaic virus* (KGMMV). Enzyme-linked immunosorbent assay (ELISA) previously used as the inspection method for such viruses reportedly has many limitations such as detection

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sensitivity and false positive reactions [2, 5, 16]. Due to these problems, researchers have developed the reverse transcription (RT)-polymerase chain reaction (PCR) method [3, 12, 19] and immunocapture RT-PCR [11] for the detection of SqMV, CGMMV and KGMMV. However, the current PCR inspection methods have different temperature cycling conditions, so their use in frontline quarantine sites for simultaneous inspection of several pathogens is inconvenient [5]. Therefore, RT-PCR and nested PCR methods with the same conditions that can be applied in quarantine sites have been reported [7-10]. Recently, the nested PCR inspection method was introduced for use in quarantine sites to improve the detection sensitivity or verify RT-PCR for the detection of seed-transmitted viruses from imported seeds [5]. However, nested PCR quarantine diagnostic systems for SqMV, CGMMV and KGMMV that can be used in quarantine sites in Korea have not yet been reported. Therefore, we developed a quarantine diagnostic system that includes RT-PCR, nested PCR and genetically modified positive control plasmids, which lead to prompt quarantine diagnosis of the 3 seed-transmitted viruses (SqMV, CGMMV and KGMMV) designated as domestic quarantine inspection target pathogens for the largest import volume seed in Korea *i.e., Cucurbitaceae* seeds.

SqMV, CGMMV and KGMMV, which were the targets of the inspection method development and the RNA or cDNA of the *Tobacco mosaic virus* (TMV), *Odontoglossum ringspot virus* (ORSV), *Pepper mild mottle virus* (PMMoV), *Ribgrass mosaic virus* (RMV), *Tobacco mild green mosaic virus* (TMGMV), *Tomato mosaic virus* (ToMV*), Cucumber mosaic virus* (CMV), *Watermelon mosaic* 2 *virus* (WMV2), *Zucchini yellow mosaic virus* (ZYMV), *Arabis mosaic virus* (ArMV), *Cherry leaf roll virus* (CLRV), *Cowpea mild mottle virus* (CPMMV), *Grapevine fanleaf virus* (GFLV), *Melon necrotic spot virus* (MNSV), *Prune dwarf virus* (PDV*), Tobacco rattle virus* (TRV), *Tomato black ring virus* (TBRV), *Cowpea severe mosaic virus* (CPSMV), *Broad bean wilt* 2 *virus* (BBWV2), *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Raspberry ringspot virus* (RpRSV), *Tomato black ring virus* (TBRV) and *Tomato spotted wilt virus* (TWSV) used in the experiment were purchased from a Bione enterprise (Korea) and collected from relevant organizations such as the Rural Development Administration and the National Academy of Agricultural Science.

In the primer design for the development of the diagnostic system, the SqMV, CGMMV and KGMMV sequences were collected from the National Center for Biotechnology Information. In the case of SqMV, for RNA2, a SqMV nearly complete sequence (AB054689), the SqMV RNA-2 strain Arizona polyprotein 2 gene (AF059532) and the SqMV RNA-2 strain Kimble polyprotein 2 gene (AF059533) were collected; and in the case of CGMMV, a complete genome (D12505) was collected. In the case of KGMMV, a complete genome (NC_003610), an isolate YM complete genome (AB162006) and a strain Yodo complete genome (AB015145) were collected; and for the sequence of the reference strain, the *Cucumber fruit mottle mosaic virus* (NC_002633), *Zucchini green mottle mosaic virus* (NC_003878), *Bean pod mottle virus* coat protein gene (M62738) and *Cowpea mosaic virus* middle-component RNA (X00729) were collected. The sequences of the collected viruses were aligned using BioEdit (Hall, 1999) and species-specific sequences were searched for at the 51-59? annealing condition using the DNAMAN software package version 6.0 [9, 14].

For the species-specific primers, SqMV (4 forward and 5 reverse primers), CGMMV (8 forward and 7 reverse primers) and KGMMV (6 forward and 4 reverse primers) were searched (Supplementary Table S1); and 10 sets, 17 sets and 15 sets that were capable of PCR amplification were combined, respectively (Supplementary Table S2). The designed primers were synthesized by Bioneer (Korea).

RNA extraction from the samples, cDNA synthesis, RT-PCR, nested PCR, electrophoresis and the selection of the quarantine diagnostic primers were carried out using previously described methods [6, 10]. To develop a quarantine diagnostic method that is the same as the existing quarantine inspection method, 2 RT-PCR primer sets were selected for each virus, as previous described [6-10]. The end-products of amplified SqMV were 680 and 852 bp; the end-products of amplified CGMMV were 447 and 609 bp; and the end-products of amplified KGMMV were 525 and 780 bp. They were selected as the final species-specific RT-PCR primer sets (Fig. 1). For the nested PCR primer, SqMV [680 bp (→567 bp) and 852 bp (→680 bp)], CGMMV [447 bp (→301 bp) and 609 bp (→372 bp)] and KGMMV [525 bp (→197 bp) and 780 bp (→430 bp)] were finally selected (Fig. 1 and Table 1).

On the other hand, it was difficult to use a virus relevant to quarantine as a control during the experiment. Hence, a genetically modified positive-control plasmid was manufactured. For the development of a positive control plasmid,

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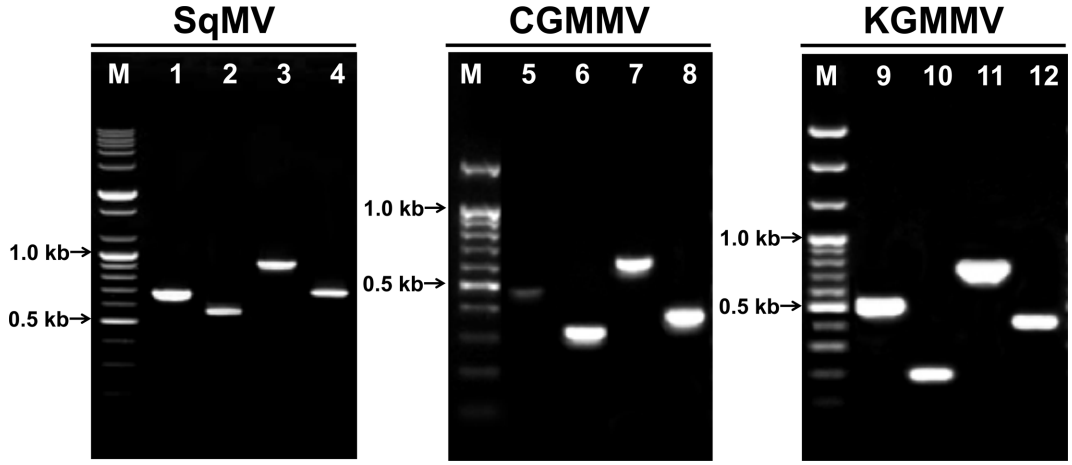

Fig. 1. Results of final selectived RT-PCR and nested-PCR for the detection of SqMV, CGMMV and KGMMV in *Cucurbitaceae*. Lane M, 100 bp step DNA Ladder; lane 1, SqMV final selected primer set #4 (680 bp); lane 2, SqMV nested-PCR product from primer set #4 (567 bp); lane 3, SqMV final selected primer set #3 (852 bp); lane 4, SqMV nested-PCR product from primer set #3 (680 bp); lane 5, CGMMV final selected primer set #11 (447 bp); lane 6, CGMMV nested-PCR product from primer set #11 (301 bp); lane 7, CGMMV final selected primer set #14 (609 bp)(Data from Park *et al*., 2011); lane 8, CGMMV nested-PCR product from primer set #14 (372 bp); lane 9, KGMMV final selected primer set #10 (525 bp); lane 10, KGMMV nested-PCR product from primer set #10 (197 bp); lane 11, KGMMV final selected primer set #12 (780 bp); lane 12, KGMMV nested-PCR product from primer set #12 (430 bp).

Table 1. Final selected primer sets for detection of SqMV, CGMMV and KGMMV

Virus	Final selection primer sets			Nested primer sets		
	Upstream	Downstream	Length (bp)	Upstream	Downstream	Length (bp)
SqMV	SqM-N20	SqM-C40	680	SqM-N30	SqM-C45	567
	SqM-N10	SqM-C40	852	SqM-N20	SqM-C40	680
CGMMV	CGMM-N40	CGMM-C50	447	CGMMN-N40	CGMMN-C50	301
	CGMM-N30*	CGMM-C60*	609*	CGMM-N35	CGMM-C70	372
KGMMV	KGMM-N55	KGMM-C59	525	KGMM-N551	KGMM-C57	197
	KGMM-N55	KGMM-C63	780	KGMM-N56	KGMM-C59	430

*Data from Park *et al*. [15].

cloning was performed using the PCR amplification product that includes all the selected RT-PCR primer sets, as the insert DNA with the use of the pGEM®-T easy vector (Promega, USA). Subsequently, using the Site-directed Mutagenesis Kit [13], a sequence (CTCGAG), with the restriction site for enzyme *Xho* I was inserted into a selected part of the nested PCR amplification product [10]. If the PCR product was cut into 2 bands by restriction enzyme *Xho* I or if the 6 inserted nucleotides were found during the sequencing, the product was considered contaminated by the genetically modified positive control plasmids developed in this study (Supplementary Fig. S1) [10].

The PCR quarantine diagnostic system developed in this study (RT-PCR, nested PCR and modified positive-control plasmid) was used in quarantine sites for inspection of SqMV, CGMMV and KGMMV from seeds and plants. Using the intranet of the Animal and Plant Quarantine Agency i.e., Pest Information System (http://10.110.128.100), the SqMV, CGMMV and KGMMV detected from *Cucurbitaceae* crops exported to Korea between 2010 and the first half of 2014 were analyzed.

The developed quarantine diagnostic system was used to inspect *Cucurbitaceae* crops exported to Korea via cargo (air, ship, railroad and land), mail and carry-on baggage between 2010 and the first half of 2014. Total 47 positive cases of SqMV were detected (2 cases in 2010, 18 cases

in 2011, 11 cases in 2012, 3 cases in 2013 and 13 cases in the first half of 2014); 67 cases of CGMMV (30 cases in 2010, 22 cases in 2011, 4 cases in 2012, 6 cases in 2013 and 5 cases in the first half of 2014); and 17 cases of KGMMV (13 cases in 2010 and 4 cases in 2011). All crops with contaminant viruses were discarded or returned.

The newly developed RT-PCR and nested PCR inspection methods and the genetically modified positive control plasmids for plant quarantine seed-transmitted viruses are expected to be in routine use and contribute to future plant quarantine.

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국문초록

박과류 관련 종자전염 검역바이러스 3종의 PCR 진단시스템 개발

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박과는 스쿼시, 호박, 애호박, 조롱박, 오이 및 수박 등 100여 속이 넘는 속이 포함된 식물과이며, 종자의 수입량이 매우 많은 작물이다. 이들이 우리나라로 수입될 때, 규제바이러스인 *Squash mosaic virus* (SqMV), *Cucumber green mottle mosaic virus* (CGMMV) 및 *Kyuri green mottle mosaic virus* (KGMMV)에 대하여 검역을 수행한다. 본 연구에서는 SqMV, CGMMV 및 KGMMV를 신속·높은 감도로 검출할 수 있는 특이적 RT-PCR 및 nested PCR 프라이머 조합과 유전자변형-양성대조구 플라스미드를 개발하였다. 또한 본 연구에서 개발한 진단시스템을 2010년부터 2014년 상반기까지 현장적용하여, SqMV 47건, CGMMV 67건 및 KGMMV 17건을 검출하였다.