Development and Optimization of a Reverse Transcription Hemi-Nested PCR Primer for the Detection of *Potato Mop-Top Virus* at Quarantine Inspection Sites in Korea

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Abstract *Potato mop-top virus* (PMTV) is classified as a plant quarantine virus in Korea. It is tested at import inspection sites. In this study, two sets of reverse transcription-hemi-nested polymerase chain reaction primers were developed to diagnose PMTV. In addition, modified-positive control plasmid was developed for the identification of false positive results in plant quarantine. The assay is expected to be useful for improving the detection and diagnosis of PMTV.

Keywords Hemi-nested polymerase chain reaction · *Potato mop-top virus* · PMTV · Quarantine

Potato is one of the major food crops in the world [1]. In 2012, 365 million tonnes (MT) of potatoes were produced from 19.2 MHa (2012 data from FAOSTAT). Potatoes are traded world-wide between countries as consumption, seed, and proceed. Although many factors may affect the risk of potato trade (ISPM 11), the greatest risk is planting materials [2]. According to the Pest Information System

(PIS) of the Animal and Plant Quarantine Agency of Korea, past decade quarantine inspection of potatoes in Korea has been tightened [3]. Many plant viruses can infect potatoes [4]. Currently, 16 viruses are listed as quarantine pests of potato by Korea. *Potato mop-top virus* (PMTV) has never been reported in Korea [5]. Enzyme-linked immunosorbent assay (ELISA) has been used since 1977 [6] to test many latent plant viruses [7, 8]. However, the disadvantages of ELISA [9, 10] in quarantine viruses make it unreliable for the detection and accurate diagnosis of quarantine viruses [7, 11]. To speed the diagnosis and shorten the inspection period, polymerase chain reaction (PCR) method has been widely used [12–16]. The use of PCR to diagnose PMTV has been reported [17–19]. However, PCR assays have different mixtures and cycling conditions by different researchers. It is inconvenient to use them in quarantine sites where many kind pathogens from various crops need to be test [20]. RT-PCR and nested PCR methods with the same reaction conditions for use at quarantine inspection sites in Korea have been reported [20–24]. In the current paper, conventional RT-PCR and hemi-nested PCR assays were developed to detect PMTV. A modified positive-control plasmid was also produced.

RNA or cDNA of PMTV and reference viruses [*Andean potato latent virus* (APLV), *Andaen potato mottle virus* (APMoV), *Arracacha virus B* (AVB), *Cucumber mosaic virus* (CMV), *Pepino mosaic virus* (PepMV), *Potato virus A* (PVA), *Potato virus X*, (PVX), *Tobacco mosaic virus* (TMV), *Tobacco ringspot virus* (TRSV), *Tobacco rattle virus* (TRV), *Tobacco streak virus* (TSV), *Tomato black ring virus* (TBRV), and *Tomato spotted wilt virus* (TSWV)] were purchased from Bi-One (Korea) or used with the help of relevant organizations. Healthy potatoes were purchased locally. RNAs of healthy potatoes, PMTV, and 13 reference viruses were extracted using RNeasy® Plant mini kit

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(Qiagen, Netherlands) [25]. Genomic DNA was extracted from healthy potatoes using DNeasy® Plant mini kit (Qiagen) following the manufacturer's instructions. RNAs of PMTV and the 13 reference viruses were dispensed in 100 µl of total plant RNA. A tenfold dilution from 10^{-2} to 10^{-9} was produced and used as template for RT-PCR and nested PCR.

Nucleotide sequences for five PMTV isolates (Genebank accession numbers AJ243719, D16193, AM503611, AY196094, DQ102381) and other *Pomoviruses* (*Beet virus Q* (AJ223597), *Broad bean necrosis virus* (D86637), and *Beet soil-borne virus* (U64512)) were obtained from the National Center for Biotechnology Information (NCBI). Five PMTV specific PCR primer sets were designed using DNAMAN software package ver 6.0 under annealing conditions of 51–59 °C [11, 26, 27]. After designing the PMTV-specific primers, a 800 bp-region of the PMTV genome (AJ243719) was synthesized (Bioneer, Korea) and quantified (1 ng/µl). The five PMTV primer sets (Supplementary Table S1) were evaluated using RT-PCR in 15 combinations (Supplementary Table S2) to test their specificity and selectivity using genomic DNA extract from healthy potatoes and tenfold dilution series of the produced plasmid (1 ng/µl). Primer combinations 2–14 showed expected products of 233–793 bp (Fig. 1a). The specificity of the 2–14 primer combination tested against 13 reference

viruses showed that 6, 7, 10, 12, and 13 did not produce a product (Gels not shown). Of these 5 primer sets, primer set 7 produced a non-specific product with genomic DNA of healthy potatoes (Fig. 1b). Therefore, it was not evaluated further. Using the PMTV plasmid, the sensitivity of primer sets 6, 10, and 13 was 10^{-7} and that of primer set 12 was 10^{-6} (Gel not shown). PMTV RT-PCR and hemi-nested PCR primer set 10 [PMTV_F3/R3 (393 bp) → PMTV_F3/R4 (276 bp)] and 13 [PMTV_F4/R2 (241 bp) → MTV_F5/R2 (126 bp)] were selected as the optimal primer sets for further evaluation (Fig. 2).

RT-PCR was performed at 42 °C for 60 min, 95 °C for 10 min, 35 cycles of 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 5 min [21]. The PCR products were separated by 1.2 % agarose (Sigma) gel electrophoresis. Primers that could re-amplify from the RT-PCR amplification products were used for nested PCR assay. The PCR conditions were the same as those for the RT-PCR reactions. Nested PCR products were analyzed by 1.2 % agarose gel electrophoresis [25]. The sensitivity, specificity, repeatability, and reproducibility of two RT-PCR and hemi-nested PCR assays were validated. Except dried potatoes, hemi-nested RT-PCR was at 10–100 times more sensitive than the first round RT-PCR for all matrices (Supplementary Table S3). PMTV specific products were observed from all RNA mixtures (Supplementary Fig. S1).

A modified-PMTV positive control plasmid was designed to contain sequence CTCGAG that could be cut by *Xho* I restriction enzyme [28]. The sequence was inserted at the position of hemi-nested PCR amplifiable region. This modified-PMTV positive could be used to identify laboratory contamination of samples as positive

Fig. 1 Selection of specific primer sets for detecting PMTV Specific selection of PMTV (a) and non-specific selection of potato genomic DNA (b) for PMTV RT-PCR primer set. Lane M, 100 bp step DNA Ladder maker (Genepia, Korea); lane number, RT-PCR primer sets for detection of PMTV. Dot, selected RT-PCR primer set

Fig. 2 Results of RT-PCR and nested PCR for detecting PMTV using HT DNA LabChip® Kit ver. 2 Lane M, HT DNA 5 K maker (Caliper Co.); lane 1, PMTV RT-PCR set 10 (F3/R3, 393 bp); lane 2, nested PCR of PMTV RT-PCR set 10 (F3/R4, 276 bp); lane 3, PMTV RT-PCR set 13 (F4/R2, 241 bp); lane 4, nested PCR of PMTV RT-PCR set 13 (F5/R2, 126 bp)

Fig. 3 *Xho* I restriction enzyme digestion of mutation-positive plasmid (triplicate experiments)

control. Using the nested set 10 (F3/R3: F3/R4), the produced positive control PCR product could be cut by the restriction enzyme *Xho* I (Fig. 3).

Detection methods should be sensitive, specific, repeatable, reproducible, and easy to carry out [29]. A two-stage PCR method (RT-PCR and hemi-nested PCR) with high detection sensitivity was selected as the detection method for use at quarantine inspection sites. The positive control can also be used to verify positive control contamination from sample testing of PMTV.

Standardization of assay conditions is also very important, particularly when many different pests have to be carried out. These single-condition RT-PCR and nested PCR quarantine diagnosis systems are very easy to use. They will contribute to the improvement of virus detection in Korea at quarantine inspection sites [7, 11]. However, these methods require >10 h for results. Therefore, they are inconvenient for field use where rapid inspection and subsequent quarantine are needed. Plant quarantine diagnostic system developed for PMTV in this study will lead to improvement in disease diagnosis and detection.

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