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Biological, Physical and Cytological Properties of *Pepper mottle virus*-SNU1 and Its RT-PCR Detection

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A strain of *Pepper mottle virus* (PepMoV) was isolated from chili pepper plants in Korea. In host range study, this virus, designated PepMoV-SNU1, shared most characteristics with PepMoV isolates reported previously. Thermal inactivation point (45°C to 75°C) and dilution end point (10^{-1} to 10^{-4}) of PepMoV-SNU1 showed differences depending on the propagation hosts. Cylindrical and pinwheel-shaped inclusions were always observed in pepper leaf tissues infected with the virus alone. Unexpectedly, a special structure of pinwheel shaped inclusion surrounded with unknown small spots was also observed in the leaf section when co-infected with a strain of pepper mild mottle virus. The partial sequence of coat protein gene and 3' untranslated region of PepMoV-SNU1 showed 98% identity with those of other PepMoV isolates. A primer pair derived from 3' end of the coat protein gene and poly A tail regions were designed. Optimal detection condition of PepMoV-SNU1 by RT-PCR was tested to determine appropriate annealing temperature and additional volumes of oligo-dT (18-mer), dNTP, and *Taq* polymerase. Under the optimized condition, an expected 500 bp PCR-product was detected in pepper leaves infected with PepMoV-SNU1 but not in healthy plants.

Keywords : Chili pepper, *Pepper mottle virus*, RT-PCR

Fifteen potyviruses occur in peppers cultivated worldwide (Abdalla et al., 1991; Kim and Choi, 2001). Even though *Tobacco etch virus* and *Potato virus Y* (PVY) are the most common in greenhouse and field grown pepper crops (Abdalla et al., 1991), *Pepper mottle virus* (PepMoV) is often considered as a causal agent for inhibition of plant growth and loss of fruit yield (Yoshihiro et al., 2003; Rodriguez-Alvardo et al., 2002).

PepMoV is transmitted by several aphid species in a non-persistent manner and has properties distinct from pepper strains of PVY in biology, serology, and genomic sequence (Hiebert and Purcifull, 1992; Vance et al., 1992). PepMoV has been of concern as the most frequently detected potyvirus in South Korea ever since the first report in 1991 (Im et al., 1991). Although this pathogen alone is not a problematic agent as cucumber mosaic virus and PVY pepper strains for Korean chili pepper industry, PepMoV has a potential of playing an important role for severe loss of the fruit yield when *Pepper mild mottle virus* (PMMoV) coinfects pepper with PepMoV.

Some isolates of PepMoV were reported in several states of USA, as well as in Korea and Japan (Purcifull et al., 1975; Im et al., 1991; Yoshihiro et al., 2003). PepMoV-FL among these isolates is well characterized in biological properties and genome sequence (Purcifull et al., 1975; Warren and Murphy, 2002), whereas PepMoV–CA, another well-characterized isolate, is known to be no longer available for further study (Warren and Murphy, 2002). Sequence data of coat protein gene or 3' nontranslated region of PepMoV isolates are recently reported in South Korea (Warren and Murphy, 2002). But their biological properties and appropriate detection conditions are not available.

For the past ten years, several laboratories including ours have continuously studied resistance of pepper plants against PepMoV through molecular mapping. Further extensive studies in biological properties and appropriate detection technique of PepMoV are required to obtain more informative data for the mapping research.

Therefore, the purposes of this study were to characterize more details of the biological, physical, and cytological properties of PepMoV-SNU1 isolated from South Korean chili pepper and to determine the optimal condition of RT-PCR for detecting the virus in pepper plant.

Materials and Methods

Virus Isolation. Infected leaves were collected from a chilli pepper (*Capsicum annuum*) plant showing severe mosaic symptom in a pepper field in the vicinity of Eumsung County, South Korea. One gram of the leaves was homo-

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genized in extraction buffer, 0.1 M Tris-HCl buffer (pH 7.2) supplemented with 1% sodium sulfite. The supernatant after centrifugation at 7,000 rpm for 10 min was gently rubbed with cotton sticks on two young leaves of *Nicotiana tabacum* cv. Xanthi-nc which were previously dusted with Carborundum (600 mesh). At 20 days after inoculation, the upper leaves from Xanthi-nc tobacco showing severe mosaic and systemic necrosis symptoms was collected and used to prepare the inoculum for the next study. Through two successive passages on *Chenopodium amaranticolor*, the necrotic lesions on inoculated leaves were individually excised and reinoculated to healthy *N. tabacum* cv. Xanthi-nc for virus propagation and maintenance.

Inoculation. Inoculum was prepared by triturating 10 g of leaves of systemically infected *N. tabacum* cv. Xanthi-nc in 50 ml of extraction buffer. The filtrate of the homogenate, after passage through four layers of cheesecloth, was applied as described above to the leaves of *Capsicum annuum*, *Chenopodium* sp., *Datura stramonium*, *Gomprena globosa*, *Nicotiana* sp., *Solanum esculentum* cultivars, *Physalis floridana*, *Petunia* hybrida and *Tetragonia expansa*. The inoculated plants were grown in a green house at 22°C-28°C. At 3 weeks post-inoculation, symptoms and virus infection were checked with naked eyes and by ELISA, respectively.

Dilution end point and thermal inactivation point. At 20 days after inoculation, leaf extracts were prepared by triturating 2 g each tissue from 'EarlyCal Wonder 123R' and 'Hung-nong kkuari' peppers, KY-57 and Xanthi-nc tobacco plants, and petunia hybrid in equal volume of distilled water with a mortar and pestle, and filtered through double layers of paper towels. The original extracts were diluted 10 fold stepwise in distilled water and used as inocula for dilution end point test. For thermal inactivation test, 5-folded dilutions of the original extract were divided into 1 ml. Each separated sample was incubated in water bath at 5°C intervals for 10 min and cooled in ice. Each inoculum was applied to inoculate two leaves per *C. amaranticolor* as described above.

Electron microscopy. At 20 days after inoculation, leaf tissues infected with PepMoV-SNU1 alone or in combination with pepper mild mottle virus (Han et al., 2001) were cut into small pieces (5 × 1 × 1 mm) and fixed in 2.5% glutaraldehyde in Millonig's phosphate buffer (MPB), pH 7.0 for 90 min at 4°C. The samples were then washed three times with MPB, and postfixed in 2% osmium tetroxide for 90 min. After three times rinsing with distilled water, the tissues were subject to uranylacetate (1.0%) staining, dehydration in 50~100% ethyl alcohol series, and embedded in Spurr resin. Ultrathin sectioning of 80nm thickness was performed on an ultramicrotome with a glass knife. Double staining was conducted in 2% uranylacetate for 20 min and 0.5% lead citrate for 10 min. Sections were observed with an electron microscope (Hitachi H-800) operating at 80 kV.

RNA extraction. RNA templates for RT-PCR were prepared from infected or noninfected peppers as follows; two leaf discs cut off with the lid of 1.5 ml microcentrifuge tube were ground with a plastic stick in 200 μL of extraction buffer (0.1 M Tris-HCl, pH 8.0, 2% SDS, 2 mM EDTA). The extract was mixed with an equal volume of TE-saturated phenol (TE; 10 mM Tris- HCl, 1 mM EDTA, pH 8.0) and once with chloroform/isoamyl alcohol (24:1 vol/vol). The upper aqueous phase after centrifugation was transferred to a new tube and mixed with 2 volumes of cold ethanol. Viral RNA was precipitated with centrifugation and washed with 80% ethanol. The resulting pellet was washed with 1 ml of 70% ethanol, drained, dried under vacuum, and then resuspended in 50 μL of diethyl pyrocarbonate (DEPC)-treated water.

The first cDNA synthesis and PCR conditions. Reverse transcription was performed using oligo dT (18-mer) in a 20 μL volume as described in the manufacturer's protocol (Promega, Southampton, UK). The first strand cDNA was diluted 5 fold with distilled water and used as a template for PCR amplification. PCR reaction mixture commonly used for each test was consisted with 1 μL of 10 x PCR buffer (TaKaRa Korea Biomedical Inc., South Korea), 1 μL of 10 mM dNTP mixture (TaKaRa Korea Biomedical Inc., South Korea), 0.4 μL of 5 U/μL *Taq* polymerase (Roche, Indianapolis, USA), and 2 μL of 10 pmol/μL each primers. Primers (sense; 5'-TGGGTCTGGCTCG-ATACGCATTTGA-3', antisense; 5'-CTCGAGTTTTTTT-TTTTTTTTTTT-3') complementary to respective 9148-9172 and 9635-9640 plus poly A tail regions of the California isolate of pepper mottle virus (PepMoV-Cal: Accession Number; NC_001517) were designed using the Primer 3 software (Genetics Computer Group Inc., Madison, WI). The final reaction volume was adjusted to 20 μL with sterilized water. The PCR was carried out on PCR machine (Peltier Thermal Cycler - MJ Research, PTC-200, USA) at the following parameters: denaturation for 3 min at 90°C, 35 cycles of denaturation for 1 min at 90°C, annealing for 1 min at 57°C and primer extension for 1 min at 72°C, and followed by one final cycle of extension for 5 min at 72°C. PCR products were analyzed by electrophoresis through a 0.8% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

Table 1. Symptomatic reactions induced by PepMoV-SNU1 on *Capsicum* spp. and other assay hosts

Assay host	Symptomatic reactions[a]
Capsicum annuum 'Chilsungcho'	SL/VC, LC, sM, Y
C. annuum 'Creolo de Morales 334'	–/–
C. annuum 'ECW 123R'	SL/VC, LC, sM
C. annuum 'Habanero'	SL/VC, LC, sM
C. annuum 'Hung-nong kkuari'	SL/VC, LC, sM, Y
Chenopodium amaranticolor	CS/–
Chenopodium quinoa	–/–
Datura stramonium	–/–
Gomprena globosa	–/–
Lycopersicon esculantum Pepe	SL/VC, mM
L. esculantum Seokang	SL/mM
Nicotiana benthamiana	SL/VC, LC, M
N. debnyei	SL/M
N. glutinosa	SL/VC, (M)[b]
N. sylvestris	SL/VC, M
N. occidentalis	SL/VC, SN
N. tabacum Blight Yellow	SL/M, SN
N. tabacum Burley	SL/VC, LC, sM
N. tabacum KY-57	VN/mM, (M)
N. tabacum Samsun 'nn'	VN/mM, (M)
N. tabacum Samsun 'NN'	SL/VC, mM
N. tabacum Turkicy	SL/mM
N. tabacum cv. Xanthi	SL/VC, mM
N. tabacum cv. Xanthi-nc	SL/VC, LC, M
Physalis floridana	SL/M, MF
Petunia hybrid	SL/VY, M
Tetragonia expansa	–/–

[a]CS, chlorotic spot; LC, leaf curling, M, mosaic; mM, mild mosaic; sM, severe mosaic; SN, systemic necrosis; SL, symptomless local infection; VC, vein clearing; VN, vein necrosis; VY, vein yellowing; Y, yellowing –; no infection.
[b]Characters in parentheses indicate occasional occurrence.

Sequence analysis. The DNA band of interest was purified using Gel Purification Kit (Pharmacia Biotech, Piscataway, N.J., USA), ligated into Easy pGem-T Vector (Promega), and transformed with JM109 *Escherichia coli* cell (Promega) as manufacturer's protocols. The recombinant plasmid DNA from successfully transformed cells was purified with Wizard Plus DNA purification system (Promega). The clones were auto-sequenced in both directions by the ABI PRISM 377 DNA Sequencer (Applied Biosystems, California, USA). The sequencing results of the cDNA insert were compared with other published isolates of pepper mottle virus using the CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic analyses were done using the CLUSTAL W and the NTSYS-pc 2.0 software (Rohlf, 1994). The robustness of the tree was assessed by bootstrap sampling of the multiple alignment (100 sets) using SEQBOOT.

Results

Host range. All five *Capsicum* accessions except 'SCM334' showed vein clearing in systemically infected leaves 5-10 days after mechanical inoculation with PepMoV-SNU1 and mosaic symptom 3-4 days since the first observation of vein clearing. *Chenopodium amaranticolor* was a unique host in which local lesions were formed on the inoculated leaves without systemic infection. Mild mosaic symptoms developed in the systemically infected leaves of tomato plants. All of *Nicotiana* species showed vein clearing, mosaic, and leaf curling in the upper non-inoculated leaves. Some species showed stem necrosis symptoms especially at high temperatures ranging from 30 to 35°C. *Physalis floridana* and *Petunia hybrida* showed mosaic and malformation symptoms in the systemic leaves. No infection was observed in *Chenopodium quinoa*, *Datura stramonium*, *Gomprena globosa* and *Tetragonia expansa*.

Stability in crude leaf extract. The stability and infectivity of PepMoV-SNU1 appeared differently according to the kind of hosts for propagation. The range of dilution end point (DEP) in the sap of pepper and tobacco plants tested was from 10 to 10,000 of reciprocal dilution. Thermal inactivating point (TIP) of PepMoV-SNU1 was higher in KY-57 tobacco than other plants tested. In petunia hybrid, both DEP and TIP values were the lowest (Table 2).

Ultrastructural properties. The cylindrical and pinwheel inclusions have been known as a touchstone of potyvirus infection in various plants. In *Capsicum annuum* 'Chilsungcho' or 'EarlyCal-Wonder (ECW) 123R', these inclusions were observed in the ultrathin sections from the systemic leaf infected with PepMoV-SNU1 alone. Putative helper components were also found in some of the sections (Fig. 1A). When the 'ECW 123R' pepper was co-inoculated with PepMoV-SNU1 and PMMoV, unique pinwheel inclusions were frequently found in the ultrathin sections of the systemic leaf with severe mosaic symptoms, in which small particle considered as the transverse section of

Table 2. Physico-biological characteristics of the PepMoV-SNU1 propagated in different hosts

Host	DEP[a] (-log)	TIP[b] (°C)
Capsicum annuum 'ECW 123R'	3~4	70
C. annuum 'Hung-nong kkuari'	3~4	60~65
Nicotiana tabacum KY-57	3	60~75
Nicotiana tabacum cv. Xanthi-nc	3~4	65
Petunia hybrida	1~2	45

[a]DEP, Dilution end point
[b]TIP, Thermal inactivating point

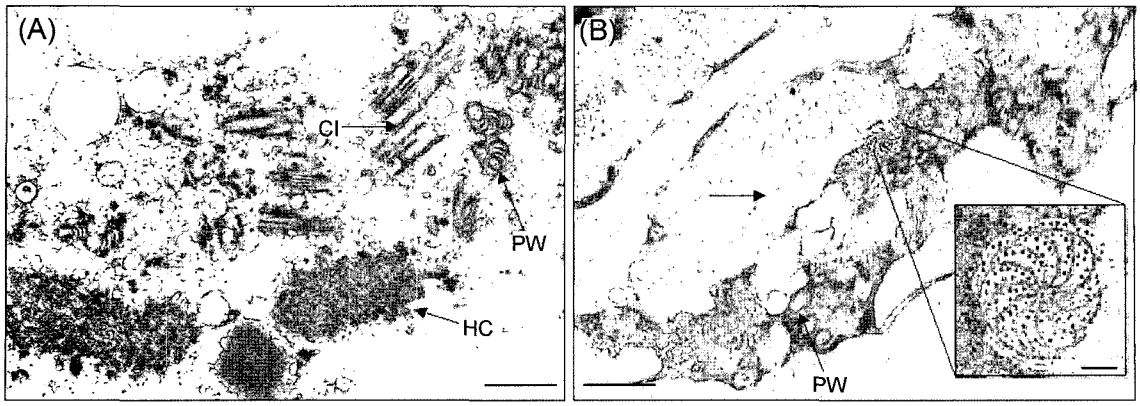

Fig. 1. Transmission electron micrographs of the ultrathin leaf sections from *Capsicum annuum* cv. 'Chilsungcho' infected with PepMoV-SNU1 (A) and *Capsicum annuum* cv. 'ECW 123R' coinfected with PepMoV-SNU1 and pepper mild mottle virus (PMMoV) (B). In panel A, cytoplasmic inclusions of cylindrical (CI) and pinwheel (PW) shapes, and helper component (HC) are shown. In panel B, PMMoV particles indicated by an arrow and pinwheel inclusions (PW) with electron-dense particles of putative PMMoV particles are shown. Inset box shows PW at higher magnification. Bars represent 50 nm.

PMMoV particles were located around the inner and outer sides of the pinwheel (Fig. 1B).

Sequence comparison and phylogenetic analysis. PCR product of a partial coat protein gene including 3'-untranslated region (3'UTR) of PepMoV-SNU1 was cloned and sequenced. Total sequence of 500 nucleotides from PepMoV-SNU1 showed 98% homology with PepMoV-CA previously reported in the USA (Vance et al., 1992). The 3'UTR of PepMoV-SNU1 was 257 nucleotides in length. Sequence comparison of the 3'UTR with those of other PepMoV isolates chosen by homology search in NCBI data bank showed that the direct repeat sequence of 76 nucleotides in PepMoV-FL (Warren and Murphy, 2003) was not present in PepMoV-SNU1 (data not shown). Phylogenetic analysis of the sequences showed that PepMoV-SNU1 and -JKK (Yoshihiro et al., 2003) formed a single cluster and were distinct from other isolates (Fig. 2).

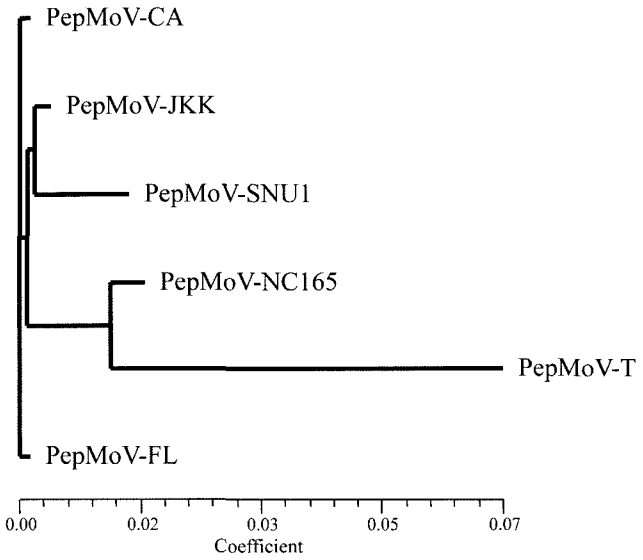

Fig. 2. Phylogenetic relationships among PepMoV isolates based on the nucleotide sequences of the 3' untranslated region. PepMoV isolates with GenBank accession number in parentheses are -CA (M96425), -JKK (AB098560), -SNU1 (AY198312), -NC165 (AF227728), -T (AF440801), and –FL (AF501591).

PCR optimization. To establish an RT-PCR condition for detecting PepMoV-SNU1 in pepper leaf, we optimized the concentrations of PCR components using the first cDNA synthesized by an RT-Kit as the template. Annealing temperature of PCR primers for specific amplification of an expected product was tested in the range from 45°C to 64.6°C with about 5°C increments. Four or five nonspecific bands were amplified with the expected band below 57°C. The specific product was amplified between 57°C and 60°C (data not shown).

With primer annealing temperature fixed at 57°C, the optimal addition volume of each PCR component was tested for practical performance. All components of the first cDNA, each primers, dNTP mixture, and *Taq* polymerase were dependent on each other. In the first cDNA titration with different addition volumes of 1, 3, 5, and 10 μL, specific amplification of the target product was achieved at the 3 μL addition, whereas no amplification was achieved at the 1 μL addition and non-specific amplifications were detected at both the 5 μL and the 10 μL additions. The selected volume of each primer for the best result was 1 μL and 2 μL, whereas increase in addition volume caused non-specific amplification and band smearing. These problems could be overcome by changing addition volumes of either dNTP mixture or *Taq* polymerase. The optimum volume of both dNTP mixture and *Taq* polymerase for PepMoV

Table 3. Optimum concentration of RT-PCR components for detection of PepMoV

Components	Addition volume (μL)	
	Tested	Optimum
RNA template (10 μg/ml)	1, 3, 5	3
Oligo-dT(18-mer) (100 pmol/μL)	1, 2, 4, 6	4
First cDNA (20 μL)	1, 3, 5,10	5
Each primer (10 pmol/μL)	1, 2, 4, 6	1
dNTP mixture (25 mM)	0.1, 0.4, 0.7, 1.0, 1.3	0.1
Taq polymerase (20 U/μL)	0.1, 0.4, 0.7, 1.0, 1.3	0.1

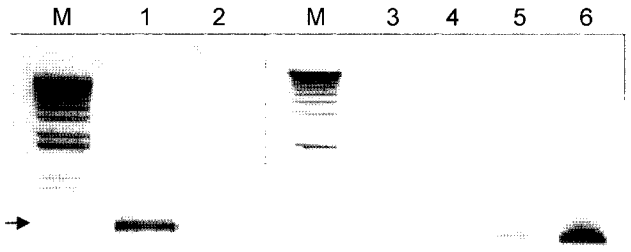

Fig. 3. RT-PCR detection of PepMoV in purified viral RNA (lane 1), PCR buffer (lane 2), and RNA extracts from healthy (lanes 3 & 4) and diseased (lanes 5 & 6) pepper leaves. Arrow indicates expected PCR product of 500 bp. M: molecular size marker.

detection was 0.1 μL, and additional amount of both components did not cause production of non-specific products and band smearing if the volume of one component was fixed at 1 μL (Table 3).

Detection of PepMoV-SNU1 by RT-PCR. Based on the optimized condition for RT-PCR, the reaction mixture was composed of 3 μL of the first cDNA dilute, 1 μL of each primers (10 pmol/μL), 0.1 μL of dNTP (10 mM), 0.1 μL of *Taq* polymerase (5 U/μL), and 14.8 μL of distilled water. The annealing temperature was 57°C. RT-PCR was carried out to detect PepMoV-SNU1 in partially purified virus and leaf samples. Specifically amplified product with the expected size of 500 bp was detected in partially purified virus and diseased leaf samples. But no PCR product was observed in buffer control and healthy leaf samples (Fig. 3).

Discussion

PepMoV has been considered as a minor pathogen threatening pepper production in South Korea (Im et al., 1991). Consequently, this virus has received less attention than other viruses for characterization of the biology and diagnosis. Currently, PepMoV has been emerged as a major pathogen threatening pepper and tomato production in South Korea. Therefore, this study was carried out to characterize an isolate of PepMoV-SNU1 in more detail and to optimize RT-PCR conditions for efficient detection of the virus.

The host range of PepMoV-SNU1 was similar to those of PepMoV-CA and –FL that were previously reported in the USA (Purcifull et al., 1975). This isolate was easily discriminated from the vein necrosis strain of potato virus Y based on the symptom of stem necrosis in *N. tabacum* cv. Xanthi-nc. The stability and infectivity of PepMoV-SNU1 were largely dependent on the propagation hosts. Petunia hybrida was not considered as an appropriate propagation host for the virus.

Ultrastructural studies showed that PepMoV-SNU1 induced scroll and pinwheel inclusions as described in other potyviruses (Kim et al., 2000; Murphy et al., 1991; Rodriguez-Cerezo et al., 1993). In a further study of the interaction between PepMoV-SNU1 and PMMoV, we found that a unique structure in the ultra-thin section from the leaf of *C. annuum* 'ECW 123R' when coinfected with both viruses. In the structure, putative PMMoV particles were regularly arranged at both inner and outer sides of the pinwheel inclusions which were produced by PepMoV-SNU1 infection. The structure was different from the pentagon or hexagon found in the cucumber when coinfected with *Watermelon mosaic virus* (WMV) and *Cucumber green mottle mosaic virus* (CGMMV) (Kim et al., 2000). To our knowledge, this structure was not reported in any previous studies with pepper as well as with other plants. Therefore, we proposed this unique structure be called as "spotted pinwheel inclusion" based on its shape. These unique structures were not detected in tissues of single infection by PepMoV-SNU1, but in tissues doubly infected by additional PMMoV.

The partial sequence analysis for 3' UTR showed that PepMoV-SNU1 was closer to Japanese isolates than to any other isolates in phylogenetic basis. The repeat sequence in 3' UTR described in PepMoV-CA (Warren and Murphy, 2003) was not found in that of PepMoV-SNU1 as well as in other isolates of PepMoV. This repeat may be considered as a unique sequence observed in PepMoV-CA alone.

Finally, we tested optimal PCR conditions for detecting PepMoV-SNU1 in pepper. Increasing the concentration of either dNTP or *Taq* polymerase did not affect the specificity for the virus detection as long as the concentration of either one component was fixed. Under this optimum condition, RT-PCR produced a specific band for detecting PepMoV-SNU1 with high-fidelity, without any non-specific bands as long as the concentration of DNA template was fixed as described in this study. The method developed in this study

should be of great utility for early and efficient detection of PepMoV in the field and in the breeding program.

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