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Characterization and RT-PCR Detection of Turnip Mosaic Virus Isolated from Chinese Cabbage in Korea

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배추에서 분리한 순무 모자이크 바이러스의 특성 및 역전사 중합효소 연쇄반응법(RT-PCR)을 이용한 검정

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ABSTRACT : Turnip mosaic virus(TuMV-Ca) was isolated from a Chinese cabbage showing severe mosaic and black necrotic spots symptoms in Korea. The virus was identified as a strain of TuMV by its host range test, particle morphology, serology, double stranded RNA analysis. For detection of the virus, reverse transcription and polymerase chain reaction(RT-PCR) was performed with a set of 18-mer TuMV-specific primers to amplify a 876 bp DNA fragment The virus was rapidly detected from total nucleic acids of virus infected tissues as well as native viral RNA of purified virion particles by RT-PCR. Detection limit of the viral RNA by RT-PCR was 10 fg.

Key words : **turnip mosaic virus, Chinese cabbage, property, RT-PCR, detection.**

Chinese cabbage (Brassica *campestris* L. ssp *pekinensis*), the most important vegetables in Korea, has been produced about 2 million tons in Korea annually. Recently, there have been severe outbreaks of mixed infection by cucumber mosaic virus(CMV) and turnip mosaic virus(TuMV) in Chinese cabbage, radish and lettuce(1). TuMV, a species of the genus *Potyvirus* in the family Potyviridae, is a one of the major viruses infecting vegetables in Korea and other countries (1, 7, 8, 9, 23). Potyviruses are mostly transmitted by several species of aphids in the nonpersistent manner. Potyviruses consist of a single filamentous particle with 680-900 nm $\times$ 12 nm in diameter. They have a single positive RNA molecule about 10 kb and one kind of structural protein (coat protein) subunit (16). The potyviral RNA contains a covalently linked protein (VPg) at the 5' terminus and is polyadenylated at the 3' terminus (16, 17). The potyvirus RNA is translated into one large polyprotein of about 346 kDa that is subsequently cleaved at specific points to produce smaller proteins by virus-coded proteases (16). To date, several isolates of TuMV have been characterized (7, 8, 9) and some of their nucleotide sequences have been determined (12, 15, 16, 17, 22). Recently, we have determined 3'-terminal region encoding the nuclear inclusion b (NIb), coat protein (CP) and 3'-noncording region of the Ca isolate of TuMV (TuMV-Ca) isolated from Chinese cabbage in Korea (GenBank AJ000690).

We report here some properties of TuMV-Ca isolated from Chinese cabbage and molecular detection of TuMV by reverse transcription and polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Virus source. TuMV-Ca used in this study was isolated from naturally infected Chinese cabbage collected in the high altitude area (600 m) of Daekwallyeong, Kangwon Province in 1992 (1). After three successive single local-lesion transfers on *Chenopodium amaranticolor*, the virus was maintained and propagated in turnip (*Brassica campestris* L. ssp. *rapifera*) plants in a temprature/light controlled glasshouse.

Host range test and physical properties. Host range of the virus was determined by mechanical inoculation of virus source in different plant species. All plants tested were evaluated by visual observation of symptoms and back inoculation to *C. amaranticolor*.

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Physical properties of the virus were assayed on *C. amaranticolor.*

Virus purification. Virus was purified from virus-inoculated turnip leaves as described previously (3). After sucrose density-gradient centrifugation (10~40% sucrose in 0.1 M phosphate buffer, pH 7.3) with a Beckman SW 27 rotor at 80,000 g for 3 hrs, the light-scattering band containing virion particles was collected and diluted with 0.01 M phosphate buffer (pH 7.3), and concentrated by centifugation at 70,000 g for 2hrs.

Electron microscopy. Purified virion particles were observed with the electron microscope. A droplet of purified virus preparation was placed on a carbon-coated grid (300 mesh) for 1 min, stained with 2% phosphotungstic acid (pH 6.8), and viewed with a JEM 100 CX-II transmission electron microscope at 80 KV. The mean length of virus particles was determined from measurements of 100 virus particles.

Serology. Gel double-diffusion test was performed in disposable petri dish containing 0.7% agarose, 0.85% NaCl and 0.02% NaN_3 in 0.02 M phosphate buffer (pH 7.3). Crude saps from virus-infected plants and purified virus preparations in 2% SDS and 0.1% 2-mercaptoethanol were boiled to 100°C for 5 min prior to its application as antigen. Crude sap from healthy plant was used as a negative control. TuMV antiserum was supplied by Dr. G. Adam (DSM, Germany). Enzyme-linked immunosorbent assay (ELISA) was carried out using a double-antibody sandwich method (18) in a 96 well microtiter plate (Immulon II, Dynatech) with alkaline phosphatase conjugate system. Absorbance was measured at 405 nm with a EL340 ELISA reader (Bio-Tek).

Viral coat protein. Purified virus preparations were boiled at 100° C for 5 min in the presence of 2% SDS and 0.2% 2-mercaptoethanol. Treated samples were separated in 10% SDS-polyacrylamide gel for 2hrs at 100 volts (13). The molecular weight marker proteins (SDS-7, Sigma) and coat protein of tobacco mosaic virus (TMV)-K strain were used as standards. Protein bands were visualized by staining the gel in Coomassie brilliant blue R-250 (18).

Viral RNA and double-stranded RNA. Viral RNA was extracted from purified virions by the method of Ryu and Park (19). RNA preparations were treated at 65°C for 15 min in the presence of 50% formamide and 10% formaldehyde, and separated in a 6% polyacrylamide gel containing 6 M urea at 50 V for 3 hrs in 89 mM Tris-borate buffer containing 2.0 mM EDTA (pH 8.0). Double-stranded RNA (dsRNA) extraction from TuMV-infected turnip leaves was conducted as described by Ryu *et al* (21) using CF-11 cellulose (Whatman). The dsRNAs of cucumber mosaic virus (CMV)-As strain were used as molecular markers. They were electrophoresed on a 1.2% agarose gel in TAE buffer at 50 V for 2 hrs. The RNA and dsRNA bands were visualized by staining gels in 100 ng/ml ethidium bromide solution and photographed under a UV transilluminator.

RT-PCR. A set of primers, 18-mer PTUCP1 (EMBL accesion number AJ001231; 5'-TCATAACCCCTTA-ACGCC-3') and PTUCP2 (EMBL accession number AJ001232; 5'-TATCACCAGGCAGGTGAG-3'), was designed to detect TuMV based on the nucleotide sequences of coat protein genes of several TuMV strains (2, 12, 15, 16). Downstream primer PTUCP1 was complementary to nucleotides 2404 to 2412 and upstream primer PTUCP2 corresponded to nucleotides 1546 to 1563 of the determined TuMV-Ca (GenBank AJ000690). The primers were designed to amplify a 876 bp DNA fragment by RT-PCR of TuMV. Reverse transcription reaction was carried out in a total 20 μl reaction volume at 42°C for 30 min in 10 mM Tris-HCl (pH 8.3) containing viral RNA, 5.0 mM $MgCl_2$, 50 mM KCl, 1.0 mM each of four dNTPs, 50 pmol PTUCP1 primer, 1 unit RNase inhibitor and 2.5 units Moloney murine leukemia virus reverse transcriptase. PCR was performed in 50 μl reaction volume containing 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 50 pmol of the two primers, 0.2 mM dNTPs and 2.5 units Taq DNA polymerase (Perkin Elmer Cetus). The PCR program was designed as denaturation at 94°C for 60 sec, annealing at 45°C for 90 sec, and polymerization at 72 °C for 90 sec for total 35 cycles, and followed one elongation step at 72°C for 10 min after the last 35th cycle. The optimum concentration of $MgCl_2$ was determined by adjusting the concentration from 0.0 to 10.0 mM in the reaction mixture of the PCR.

RESULTS

Host range and symptomatology. An isolate, designated TuMV-Ca, from a naturally infected Chinese cabbage showing severe mosaic, black necrotic spots of the leaves and dwarfing in whole plant, was used for virus source. After inoculation of the virus, the plants showed mosaic and distorted symptoms on young turnip leaves in 12 days and systemic symptoms on older leaves in 16~18 days. Systemic infections were also observed on the

Table 1. Reaction of test plants to an isolate of TuMV Chinese cabbage

Plants tested	Reactions[a]
Brassica campestris L. ssp. *pekinensis*	Y,mM/Ch,nRS
B. campestris L. ssp. *rapifera*	D,M,St/Y
B. juncea	-/-
Raphanus sativus	M,St/Ch
Chenopodium amaranticolor	-/nLL
C. quinoa	-/nLL
Nicotiana clevelandii	-/-
N. rustica	-/-
N. tabacum cv. NC82	-/-
N. tabacum cv. Samsun	-/nLL
N. tabacum cv. Xanthi	-/nLL
N. tabacum cv. Wisconsin 38	-/nLL
Citrullus vulgaris	-/-
Arachis hypogaea	-/-
Glycine max	-/-
Capsicum annuum cv. Hongilpum	-/-
Cucurbita pepo	-/-
Cucumis sativus	-/-
Lactuca sativa	-/-
Physalis floridana	-/cLL
Oenothera odorata	-/-
Datura stramonium	-/-
Vigna unguiculata	-/-
Siegesbeckia glabrescens	-/-
Zea mays	-/-
Fragaria grandiflora	-/-

[a]Format for symptom symbols: M(systemic mosaic), mM(mild mosaic), St(stunting), nRS(necrotic black ringspots), nLL(necrotic local lesion), cLL(chlorotic local lesion), Y(yellowing), Ch(chlorosis), D(distorting), -(no reaction). Denominator indicates inoculated leaf and numerator means upper leaves of inoculated part.

leaves of *Brassica campestris* L. ssp. *pekinensis, B. campestris* L. *rapifera*, and *Raphanus sativus*(Table 1). On the other hand, *Chenopodium amaranticolor, C. quinoa, Lactuca sativa, Nicotiana tabacum* cv. Samsun, cv. Wisconsin 38, cv. Xanthi, and *Physalis floridana* showed local lesions on their inoculated leaves. However, the virus did not infect other tested plants in Table 1, and these plants neither produced any symptoms by back inoculation on *C. amaranticolor* nor was virus detected by ELISA.

Properties *in vitro*. The thermal inactivation point of the virus was at 65°C, the dilution end point was 10^{-4}, and the longevity *in vitro* was 3 days on *C. amaranticolor.*

Virus purification. Virus particles were recovered from 21th fraction about 33% sucrose area after sucrose density gradient centrifugation (Fig. 1), and then concentrated the virus preparation. Purified TuMV-

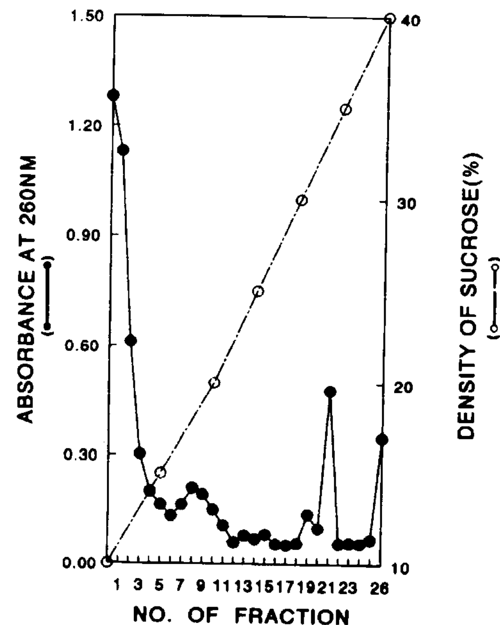

Fig. 1. Ultraviolet absorption profile of purified preparation of TuMV on sucrose density gradient centrifugation(10-40% sucrose). Volume of each fraction was 1 ml.

Fig. 2. Electron micrograph of purified TuMV particles negatively stained in 2% potassium phosphotungstate(pH6.8). Scale bar represents 300 nm.

Ca preparation showed the typical ultraviolet absorption curve of nucleoprotein with a maximum at 260 nm and a minimum at 246 nm. The mean A260/A280 ratio was 1.22. Electron micrograph of purified virus showed numerous filamentous particles, which was 745 nm in length and 11 nm in width (Fig. 2).

Serological assay. Crude sap from infected Chinese cabbage and purified preparation of TuMV-Ca strongly reacted with antiserum to TuMV in agarose gel double-diffusion test (Fig. 3). The virus could be detected by the ELISA, but no reaction was occurred

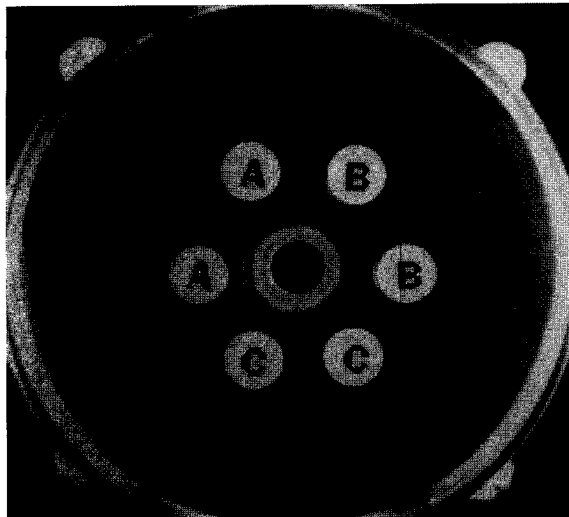

Fig. 3. Serological reaction of TuMV antiserum(central well) to purified TuMV-Ca(A), sap from TuMV-Ca infected turnip (B), and sap from healthy turnip(C).

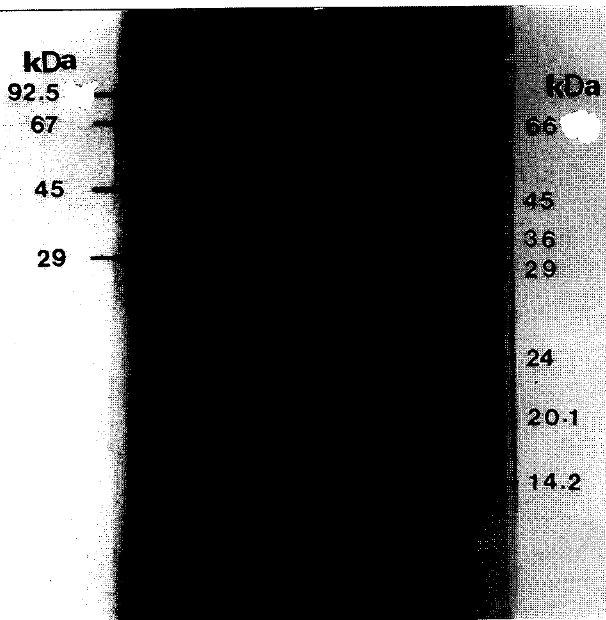

Fig. 4. Coat protein pattern of TuMV-Ca on 10% SDS-PAGE. HF and MF indicate heavy form and middle form proteins of TuMV, respectively. Lanes 1 and 4 : marker proteins ; 2 : tobacco mosaic virus(TMV) ; 3 : TuMV-Ca.

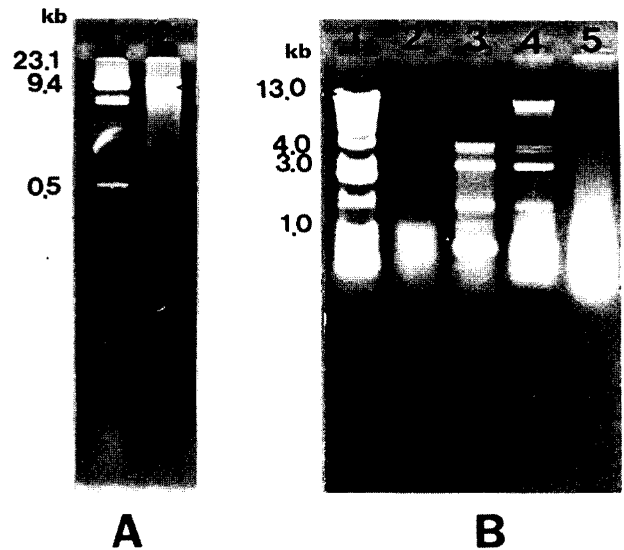

Fig. 5. Electrophoretic patterns of viral RNA(A) and dsRNA (B) of TuMV. Blank arrowhead indicate viral RNA and dsRNA of TuMV-Ca. Photo A : 1 ; Molecular weight marker, 2 ; TuMV-Ca RNA. Photo B : 1 ; Molecular weight marker, 2 ; dsRNA from TuMV-Ca infected turnip, 3 ; dsRNAs from CMV-As infected tobacco, 4 ; dsRNAs from naturally TuMV and CMV infected Chinese cabbage, and 5 ; healthy leaf of Chinese cabbage.

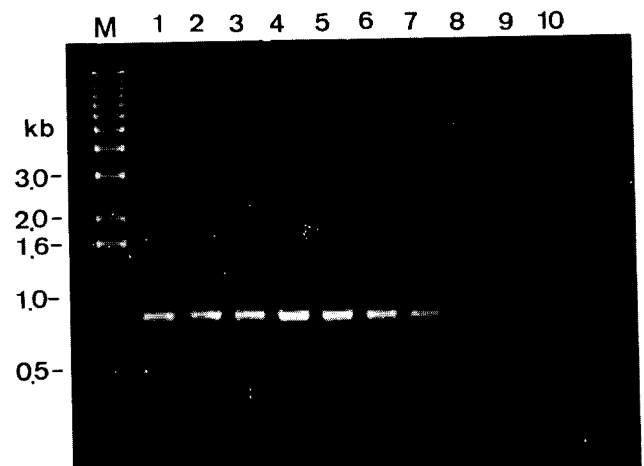

Fig. 6. Detection of purified TuMV RNA by RT-PCR. Lane M ; 1kb DNA ladder, 1 ; 100 ng, 2 ; 10 ng, 3 ; 1 ng, 4 ; 100 pg, 5 ; 10 pg, 6 ; 1 pg, 7 ; 100 fg, 8 ; 10 fg, 9 ; 1 fg, 10 ; 0.1 fg of purified viral RNA.

in crude extracts from healthy plant or purified CMV-As (data not shown).

SDS-PAGE. The coat protein of TuMV-Ca revealed electrophoretic heterogeneity, 33 kDa major protein and 26 kDa protein, by SDS-PAGE (Fig. 4). The 33 kDa protein (heavy form) was the same Mr as the viral coat protein and the 26 kDa protein (middle form) was estimated the proteolytic degraded form of the coat protein.

Viral RNA and dsRNA analyses. Yield of the purified viral RNA was 36 μg per mg of the purified virus and A260/A289 ratio was 1.92. When purified viral RNA was separated in polyacrylamide gel under urea-denaturing condition, a single band, about 10 kb was detected (Fig. 5A).

One migrating dsRNA was obtained from TuMV in-

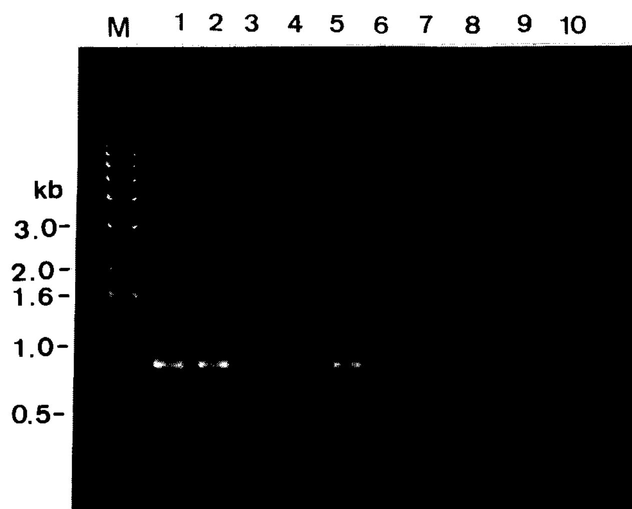

Fig. 7. Detection of TuMV by RT-PCR in crude extracts of several vegetables collected in fields. Lane M ; 1kb DNA ladder, 1,2,3,4 ; Chinese cabbages, 5,6,7 ; radishes, 8,9,10 ; lettuces.

fected plant, and the size in base pairs was the same as the viral RNA (Fig. 5B).

RT-PCR. Two conserved regions, positioned at 5' and 3' ends of the TuMV coat protein gene, were selected for design of the primers for TuMV-specific detection based upon the known nucleotide sequences of TuMV strains. Optimum concentration of the $MgCl_2$ to produce PCR product efficiently was 2.5 mM.

Single 876 bp DNA product was obtained by RT-PCR of purified TuMV-Ca RNA (Fig. 6). No product was produced from healthy plant with the same primers by the RT-PCR.

Serial dilutions of purified TuMV-Ca RNA and total nucleic acids from the virus-infected turnip leaves were examined to determine the sensitivity for detection of the virus. RT-PCR revealed to be able to detect 10 fg of TuMV RNA (Fig. 6) and 1:16,384 (w/v) dilution of the crude nucleic acids (data not shown). One microliter aliquot from 50 μl volume for 0.1 g of original leaf tissue was sufficient to produce the diagnostic PCR DNA fragment following the RT-PCR.

Extracts of total nucleic acids from all TuMV infected plants including Chinese cabbage, lettuce, and turnip gave positive results in the RT-PCR assay, while no PCR product was obtained from healthy plants (Fig. 7).

DISCUSSION

From the experimental results including pathogenic tests, serology, physical properties, coat protein, viral RNA/dsRNA assay and molecular detection the virus isolated in this study was identified as a strain of TuMV. Choi and Wakimoto (5) described a coat protein heterogeneity of TuMV in the presence of SDS as three components with molecular weights of 33 kDa, 26 kDa and 24 kDa proteins. Our results also showed two protein bands by SDS-PAGE with purified virus preparation. Electrophoretic heterogeneity has been observed with most of potyviruses when was analysed by SDS-PAGE (10, 11, 14). It was known that coat proteins of potyviruses can be degraded during purification and storage by enzymes of plant or virus origin, giving rise to faster moving components in SDS-PAGE (9, 10). Previous studies (5, 9, 10) and the present study suggest that the coat protein of TuMV in prone to relatively rapid degradation which results in two to three major bands in SDS-PAGE.

A comparison of the nucleotide sequences to certain viral genes or regions allows important information to us in identifying and classifying strains of TuMV and other viruses. The nucleotide sequences of several TuMV strains have been reported (1, 4, 12, 15, 16). By doing restriction endonuclease and nucleotide sequence analyses, TuMV-Ca was able to be distinguished from those of reported other strains (19). The 3'-noncoding region which is useful data for classifying potyviruses was highly conserved (99.5-94.3%) among TuMV strains (4, 16, 17, 22). TuMV-Ca exhibits 96.9% to 94.1% amino acid homologies of the coat protein to those of other strains (data not shown).

This study also showed that RT-PCR using the TuMV-specific primers was very useful for the diagnosis of the virus. This was routinely reproducible in separate replicational tests with the same samples. Significantly, TuMV infection was detectable by RT-PCR in samples which showed no symptoms or in samples which did not give positive results on ELISA and gel double-diffusion tests. The lowest concentration of template viral RNA required to detect the virus was 10 fg of viral RNA, and detection limit for the maximum dilution of crude nucleic acids from TuMV-infected turnip leaf was 1:16,384 (w/v) in this study. This results were similar to previously reported data with other plant viruses (6, 20). De Blas *et al* (6) reported that pepper plants contain inhibitor hampering RT-PCR detection of CMV. However, we obtained positive results with turnip plants without any inhibition. This method reveals rapid, sensitive and reproducible diagnostic tool for TuMV.

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요 약

심한 모자이크와 괴저반점병징을 보이는 배추로부터 순무 모자이크 바이러스(TuMV-Ca)를 분리하였다. 본 바이러스는 기주범위조사, 바이러스의 형태, 혈청학적 특성과 이중쇄상 RNA 분석결과 순무 모자이크 바이러스의 한 계통으로 동정되었다. 본 바이러스를 검정하기 위하여 각각 18개의 염기로 구성된 순무 모자이크 바이러스 특이적 프라이머를 사용하여 876 bp 크기의 DNA가 합성되도록 역전사 중합효소 연쇄반응(RT-PCR)을 실시하였다. 본 방법으로 순화 바이러스 RNA와 바이러스가 감염된 식물체로부터 추출한 핵산을 사용한 경우 모두에서 쉽게 바이러스를 검정 가능하였다. RT-PCR의 TuMV 검정한계는 최소 10fg의 바이러스 RNA로 조사되었다.

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