Acta Biologica Hungarica 66(2), pp. 222–230 (2015)
DOI: 10.1556/018.66.2015.2.8

MOLECULAR CHARACTERIZATION OF THE *TOBACCO RATTLE VIRUS* RNA2 GENOME ISOLATED FROM *GLADIOLUS*

YOON J. KIM, MI S. LIM, SU M. KIM, KI H. RYU and SUN H. CHOI*

Department of Horticulture, Biotechnology and Landscape Architecture, Seoul Women's University, Seoul 139-774, Korea

(Received: August 14, 2014; accepted: October 27, 2014)

Tobacco rattle virus (TRV-K) was first identified in a symptomatic *Gladiolus* plant cultivated in Korea. We analyzed the TRV-K genome and compared its phylogeny with other TRV isolates. After constructing of a full-length genomic RNA2 strand clone, a complete sequence was generated from several overlapping clones. The cloned genome was 3261 bases in length, identical to TRV-K, and had three open reading frames. TRV-K had the highest sequence identity with the American isolate TRV-ORY. Sequence analysis of the RNA2 genome showed that TRV-K contains an intact 2a, 2b, and 2c coding sequence and an RNA1-related 3′ terminus, which is typical of TRV RNA2. Phylogenetic analysis revealed that TRV-K is in the same cluster as the American isolates and another Korean isolate, TRV-SK; however, it was in a different cluster than the European isolates.

Keywords: Tobacco rattle virus – Gladiolus – Korean isolate – RNA2 – phylogeny

INTRODUCTION

The tobravirus genome is divided into two positive-sense, single-stranded RNAs (RNA1 and RNA2), which are encapsidated separately into rod-shaped particles. *Tobacco rattle virus* (TRV), the type species of this genus, infects many plant species and causes economically significant diseases in potato, tobacco, and ornamental bulbs [7, 8]. This virus is transmitted between plants by soil-inhabiting nematodes. This indicates a highly specific relationship between the virus and nematode [4, 8]. Previous studies of TRV showed that different isolates have very little sequence identity in the RNA2 region. The TRV RNA2 genome is 2–4 kb long and contains three or four open reading frames (ORFs) that encode viral coat proteins (CPs) and two or three ORFs related to the viral transmission process via nematodes [11, 13]. In the RNA2 3′ nontranslated region (NTR), partial or complete tobravirus RNA1 3′ coding sequences were present in many of the reported sequences. Both the 5′ and 3′ RNA2 NTRs from various TRV isolates show considerable size and composition differen-

*Corresponding author; e-mail address: paxs@swu.ac.kr

ces [6]. The extreme RNA2 sequence variation between different tobraviruses makes it difficult to define the complete genome organization [8]. The present study was performed to determine the RNA2 nucleotide sequence of a TRV isolated from *Gladiolus* (TRV-K) in Korea [9] and to investigate its molecular characteristics and evolutionary status compared with other isolates in the molecular taxonomy. TRV-K co-infection with *Bean yellow mosaic virus, Clover yellow vein virus, Broad bean wilt virus,* and *Cucumber mosaic virus* was observed in leaf tissues of various symptomatic gladiolus plants [9]. This is the first report characterization of this natural TRV infection identified in *Gladiolus* in Korea.

MATERIALS AND METHODS

TRV-K was originally isolated from the bulb of *Gladiolus* sp. infected once that showed notched leaves [9]. The virus source was obtained from samples deposited in the Plant Virus Genbank at Seoul Women's University by Dr. M. U. Chang (Youngnam University) [10]. To confirm the viral symptomatology, *Nicotiana* sp. were used as host plants. Viral genomic RNA was extracted from purified virus particles by SDS-proteinase-K/phenol extraction followed by ethanol precipitation [8]. First strand cDNA was synthesized with reverse transcriptase (Superscript, Roche) using the R2TRV3 oligonucleotide (5′-GAGCATGCGGGCGTAATAACGCTTACGT-3′), which is complimentary to the RNA2 3′-terminal nucleotides. The initial cDNA served as a template for full-length cDNA amplification by polymerase chain reaction (PCR) using *Taq* DNA polymerase (Roche) with an R2TRV5 upstream primer containing an SP6 promoter site (5′-GAGAGCTCATTTAGGTGACACTATAGATAAAACATTGCACCTTTGGTG-3′) and an R2TRV3 reverse primer [2]. PCR was performed in a thermal cycler (BIO-RAD, USA) under the following conditions: initial denaturation at 94 °C for 15 min followed by 5 PCR cycles of 30 s at 94 °C, 30 s at 59 °C, and 3 min at 68 °C, then 10 PCR cycles of 30 s at 94 °C, 30 s at 60 °C, and 4 min at 68 °C, 10 PCR cycles of 30 s at 94 °C, 30 s at 61 °C, and 5 min at 68 °C, and a final 10 min extension at 68 °C. PCR products containing TRV RNA2 were cloned into the pUC19 vector *Sac*I/*Sph*I site. Recombinant plasmids were amplified in *Escherichia coli* (strain JM109, Promega). The generated TRV RNA2 clone sequences and RT-PCR amplicons were confirmed based on their various endonuclease digestion sites. Selected subclones containing different cDNA fragments, generated by *Bam*HI and *Hind*III digestion, were sequenced in both directions using the dideoxynucleotide chain termination method (Bionics, Korea). Multiple sequence alignment was performed on the experimentally determined nucleotide sequence and the deduced amino acid sequences using the MegAlign program (Lasergene 6, DNASTAR Inc.). Phylogenetic and evolutionary analyses were performed using ClustalW and Mega 6 [12].

RESULTS AND DISCUSSION

Nicotiana tabacum and *N. clevelandii* were inoculated with TRV-K, and symptoms were observed after 27 and 10 days, respectively (Fig. 1A, 1B). Symptom-bearing plants were verified for TRV-K infection by RT-PCR using the TRV diagnostic primer set (forward; 5′-GATGAAATTATGTCTCTGTATCG-3′ and reverse; 5′-CCAGTATAGTACTTGTTCAATAC-3′). This produced the expected 800 bp PCR product (Fig. 1C). Full-length TRV-K RNA2 cDNA copies were amplified from viral RNA (Fig. 2A) by long-template (LT)-PCR and were cloned into an *E. coli* amplification vector (Fig. 2B). Both the full-length RT-PCR product and its recombinant cDNA clone were validated by restriction fragment length polymorphism (RFLP) analysis using *Bam*HI, *Eco*RI, *Hind*III, *Nci*I, *Pst*I, *Sac*I, *Sal*I, *Sph*I, and *Xba*I. This fingerprint

Fig. 1. Host plant symptoms of *Tobacco rattle virus* (TRV-K) infection and reverse transcription-polymerase chain reaction (RT-PCR) detection with TRV-specific primers. (A) *Nicotiana tabacum* "Samsun", (B) *Nicotiana clevelandii,* (C) Lane M, DNA size marker; Lane 1, PCR product amplified from *N. tabacum;* Lane 2, PCR product from *N. clevelandi;* Lane 3, PCR product from TRV-infected tobacco leaf; Lane 4, healthy tobacco; Lane 5, TRV-K viral RNA

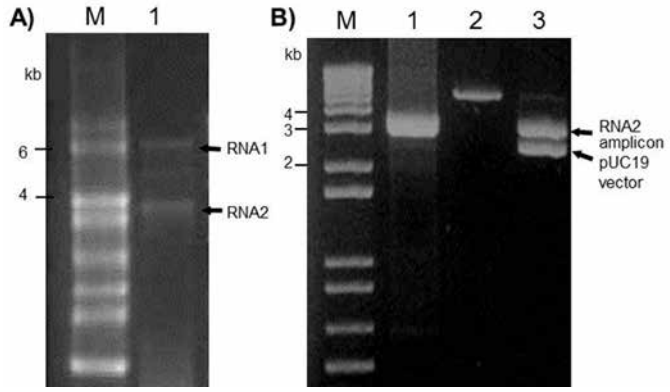

Fig. 2. TRV-K RNA1 and RNA2 electrophoresis. (A) Full-length TRV-K RNA2 cDNA clone and (B) size confirmation by digestion. (A) Lane M, RNA size marker; Lane 1, TRV viral RNA. (B) Lane M, DNA size marker; Lane 1, full-length cDNA PCR product; Lane 2, linearized full-length cDNA clone; Lane 3, digested insert cDNA and vector to confirm cloning

was compared with the calculated fingerprint derived from GenBank's TRV sequence information (Fig. 3).

The TRV-K RNA2 genome was 3261 nucleotides long. It contained three ORFs with a 22 kDa viral CP (2a), a 23 kDa protein (2b), and an 18 kDa protein (2c). In addition, there were sequences corresponding to a truncated 16 kDa protein similar to

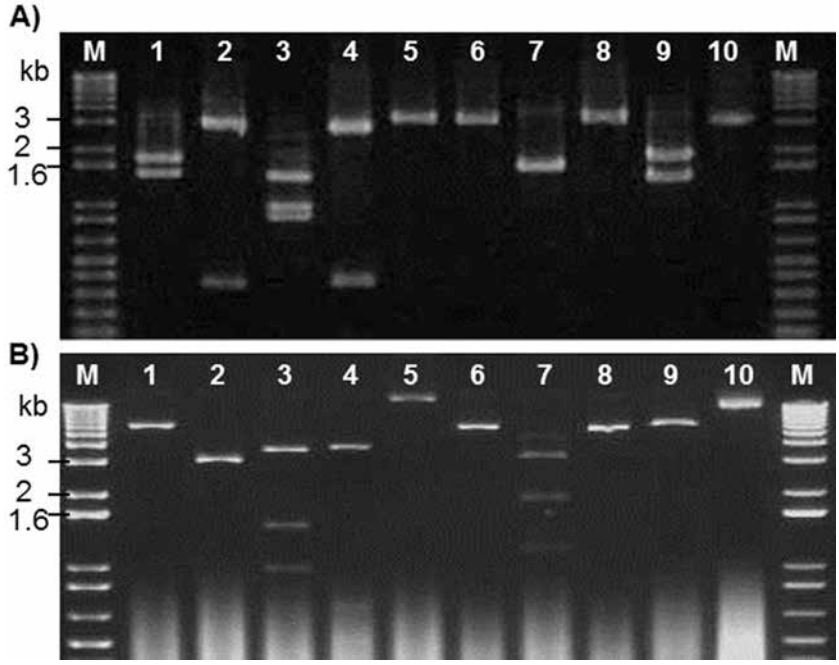

Fig. 3. Restriction fragment length polymorphism (RFLP) results of (A) the full-length cDNA PCR product and (B) the TRV-K RNA2 clone. Lane M, DNA size marker; Lane 1, *Bam*HI digest; Lane 2, *Eco*RI digest; Lane 3, *Hind*III digest; Lane 4, *Nci*I digest; Lane 5, *Pst*I digest; Lane 6, *Sac*I digest; Lane 7, *Sal*I digest; Lane 8, *Sph*I digest; Lane 9, *Xba*I digest; Lane 10, full-length PCR product (A) or clone (B)

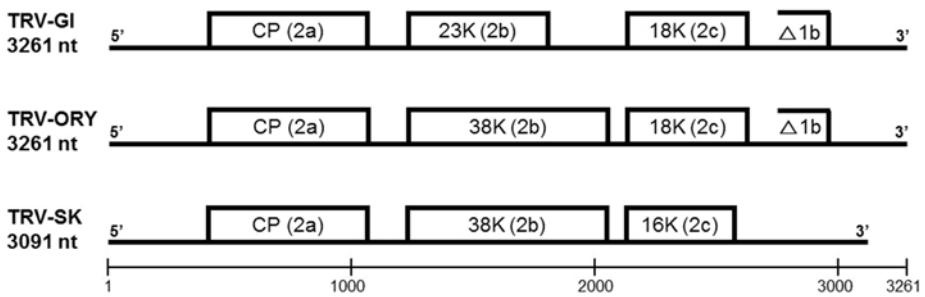

Fig. 4. Schematic representation of the TRV RNA2 genome organization from the TRV-K, TRV-ORY, and TRV-SK isolates. Boxes indicate ORFs and open boxes indicate truncated ORF. CP is the coat protein

one located at the RNA1 3′ terminus (Fig. 4). Several isolates also contained a truncated 1b gene ORF that was also situated at the RNA1 3′ terminus. This is possibly the result of RNA recombination [3]. The TRV-K 2c ORF appears to be unique. TRV-ORY also has a unique 18 kDa protein, and the virus has been reported to be nematode-transmissible [11] (Fig. 4).

The TRV-K CP gene was 603 bp (200 amino acids). This is similar to the CP gene size in other known TRV isolates, which range from 591 to 633 bp (Table 1). TRV-K RNA2 CP exhibits variable sequence identity (43.0 to 100% on the amino acid level) with the 13 known TRV isolate CPs (Table 1). The TRV-K 5′ NTR nucleotide sequence was more conserved than the 250 bp 3′ NTR partial sequences. Analysis of TRV-K and TRV-ORY revealed that CP had 100% identity at the nucleotide and amino acid levels, and the whole RNA2 sequence had 99.7% identity (Table 1). The TRV-K RNA2 molecule had a shorter 2b ORF (23 kDa) than the equivalent TRV-ORY RNA2 molecule (38 kDa). TRV-K appeared to contain a single nucleotide substitution that introduced an early stop codon (TAG), which was CAG in the TRV-ORY 2b gene (data not shown). In a previous study on TRV-Ho, this early translation cessation is hypothesized to indicate that RNA2 molecules can acquire 3′ ends of various lengths while losing large portions of their RNA2-specific sequences [7]. There were considerable differences in the RNA2 genome sizes between TRV-K and another Korean isolate TRV-SK. The TRV-SK isolate lacked the 1b-related ORF, which made it shorter than the TRV-K isolate (Fig. 4). The 2b protein, together with the 2c protein, is responsible for nematodal virus transmission [5]. Thus, it allows the virus to be acquired by specific nematode species.

Low RNA2 sequence identity was observed between TRV-K and several European isolates (Rostock, Tp01, PPk20, ON, PaY4, and TCM), with identities ranging from 29.4% to 49.4% (Table 1). The conserved TRV-K 3′ NTR (250 bp) showed 99.8% sequence identity with TRV-ORY, whereas the TRV-SK isolate showed 42.9% identity (Table 1). While the TRV-SK isolate also originated from Korea, it was obtained from potato; therefore, some variability in RNA2 genome composition exists depending on the host plant and recombination.

Phylogenetic trees of the TRV isolates and the *Pea early browning virus* (PEBV), a tobravirus outgroup, were constructed for the complete RNA2 genome sequences, both NTRs, and the CP amino acid sequences using the maximum likelihood method (Fig. 5). The trees generated were consistent with the unweighted pair group method results using both arithmetic mean and maximum parsimony comparisons (data not shown). The TRV isolates were clustered into two main groups (Fig. 5). Based on the RNA2 complete genome sequences, TRV-K was part of a cluster that included American isolates and the other Korean isolate, TRV-SK. The European isolates Ppk20, Rostock, Tp01, ON, PaY4, and TCM were phylogenetically distinct from TRV-K and clustered into a different group (Fig. 5A). The phylogeny derived from the CP analysis agreed well with the complete genome, except isolate PpK20 (Fig. 5A, 5D). The results from the 5′ NTR phylogenetic tree showed that the TRV isolates could be divided in a manner similar to the complete RNA2 genome comparison (Fig. 5B). In particular, PEBV appeared to be closely related to TCM, as observed by the

Table 1
Size comparison and sequence identity of various regions in the RNA2 molecule between *Tobacco rattle virus* (TRV-K) and other TRV isolates and one *Pea early browning virus* (PEBV) outgroup

Isolates used for comparison	CP[c] (2a)				5′ NTR[d]		3′ NTR		Complete RNA2		Accession (NCBI)
	nt[a]	%	aa[b]	%	nt	%	nt	(250bp)%	nt.	%	
TRV-K	603	100.0	200	100.0	522	100.0	514	100.0	3261	100.0	AJ536414 In this study
SK	603	98.2	200	98.5	523	97.9	280	42.9	3091	89.5	AB369280
MI-1	603	97.8	200	99.0	522	98.7	571	89.0	3685	94.8	GQ903772
ORY	603	100.0	200	100.0	521	99.2	571	99.8	3261	99.7	AF034621
OR2	603	98.5	200	98.5	522	98.9	565	96.7	3679	95.9	AY166661
BM	603	97.8	200	97.0	523	98.7	421	63.0	3536	94.5	AY166663
Cot2	603	98.8	200	99.5	523	97.9	421	63.4	3536	94.7	AY166662
Umt1	603	98.5	200	98.0	522	98.9	437	75.5	2905	87.6	AY166660
Rostock	591	51.7	196	43.0	473	53.4	255	43.1	2014	29.4	AJ272198
Tp01	591	52.2	196	43.0	474	53.6	412	59.9	3216	44.6	AJ009833
PpK20	615	51.7	204	43.0	556	58.0	400	54.3	3855	44.9	Z36974
ON	633	52.4	210	45.0	709	57.7	751	90.0	3357	44.9	Z97357
PaY4	633	53.2	210	45.0	712	58.4	255	43.8	3926	49.4	AJ250488
TCM	618	52.4	205	46.0	546	67.4	255	42.6	3389	45.2	X03955
PEBV-SP5	639	57.4	212	58.0	509	55.0	480	43.4	3374	42.0	X51828

[a] indicates number of nucleotides.
[b] indicates number of amino acids.
[c] indicates coat protein.
[d] indicates nontranslated region.

5′ NTR comparison. This finding agreed well with a previous report indicating that PEBV was closely related to TRV-TCM and other European isolates [6, 8].

The terminal 3′ noncoding sequences found in several strains are hypothesized to be derived from RNA1. According to a BLAST nucleotide search, TRV-K has sequences derived from RNA1, similar to TRV-ORY (Fig. 4). This RNA2 recombined region in many TRV isolates can have a length greater than 1 kb and can include partial or complete gene copies of RNA1-encoded 1a, a movement protein, and/or 1b, a cysteine-rich protein [1, 6]. Recombination may also occur among different PEBV tobraviruses. Therefore, the TRV-K RNA2 3′ NTR is thought to contain recombined RNA1 genes and genes encoding nematodal transmission-proteins, thus providing evidence for an evolutionary step within the natural habitat. Additionally, the 3′ NTR phylogeny showed a conserved sequence among the TRV isolates, which indicates that all the TRV isolates diverged from PEBV without significant grouping (Fig. 5C).

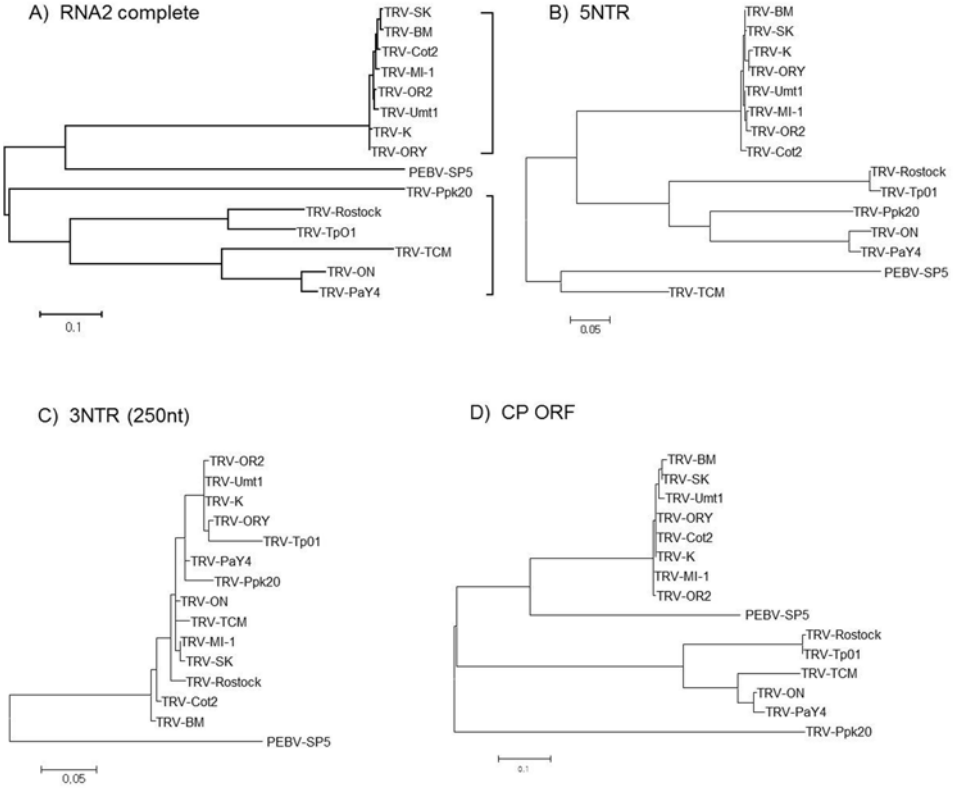

Fig. 5. Molecular phylogenetic analyses of various RNA2 regions from 14 TRV isolates and *Pea early browning virus* (PEBV), a tobravirus outgroup. Trees were calculated using the neighbor-joining method. The tree is drawn to scale with branch lengths measured as the number of substitutions per site. Evolutionary analyses were conducted using the MEGA6 program. A phylogenetic tree indicating the relationship between (A) the complete RNA2 genomes of TRV isolates and PEBV, (B) the RNA2 5′ nontranslated region (NTR), (C) the RNA2 3′ NTR, and (D) the RNA2 coat protein

TRV-K and TRV-ORY both contained an identical 3261 nucleotide RNA2 sequence. The 3′ terminal region of these isolates derived from RNA1, but differed with regard to the exact ORF 2b sequence and size. This discrepancy was also reported for Rostock and Tp01 isolates. Those isolates share 98% identity in the 5′ NTR; however, one is recombinant and has a duplicate 3′ terminal 1b gene, whereas the other is not recombinant [8]. The differing size and genetic variability of RNA2 suggest that tobraviruses possess an adaptation mechanism to infect new hosts by varying conditions at different infection stages [8]. Interestingly, the TRV-Ho isolate contains one RNA1 genome and two different RNA2 genomes. Moreover, different RNA2 species co-infecting the same *Hosta* plant were shown to have different genome sizes as a result of deletions, recombinations, and reassortments [7]. TRV-SYM, isolated in England from spinach, also showed a unique genome organization containing three unknown ORFs upstream of the CP gene [1].

Here, we have described the genome properties of recombinant TRV-K RNA2, isolated in Korea. We suggest that a specific 2b protein segment is redundant. Alternately, the 3′ terminal deletions in the 2b gene may be advantageous for the virus, particularly regarding replication. This suggestion is based on the presence of identical 3′ termini in both the RNA1 and RNA2 molecules, which is considered advantageous for virus replication [7]. Rapid TRV RNA2 composition changes during serial passages may generate various mutants with different deletions following cognate RNA1 recombination [4]. Additionally, spontaneous RNA2 deletion mutants arise rapidly after a single mechanical transfer [14]. Our next step will be to determine whether functional TRV-K 2b protein is translated at the predicted size and whether passage by mechanical inoculation will result in RNA2 deletion accumulation.

ACKNOWLEDGEMENTS

This work was supported by a young researchers grant (2014-0279) from Seoul Women's University (2014).

REFERENCES

1. Ashfaq, M., McGavin, W., MacFarlane, S. A. (2011) RNA2 of TRV SYM breaks the rules for tobravirus genome structure. *Virus Res. 160,* 435–438.
2. Choi, S. H., Ryu, K. H. (2008) Generation of infectious transcripts from Korean strain and mild mottle strain of potato virus X. *J. Microbiol. 46,* 502–507.
3. Crosslin, J. M., Hamm, P. B., Kirk, W. W., Hammond, R. W. (2010) Complete genome sequence of a *Tobacco rattle virus* isolate from Michigan-grown potatoes. *Arch. Virol. 155,* 621–625.
4. Hernandez, C., Carette, J. E., Brown, D. J. F., Bol, J. F. (1996) Serial passage of tobacco rattle virus under different selection conditions results in deletion of structural and nonstructural genes in RNA 2. *J. Virol. 70,* 4933–4940.
5. Hernandez, C., Visser, P. B., Brown, D. J. F., Bol, J. F. (1997) Transmission of tobacco rattle virus isolate PpK20 by its nematode vector requires one of the two non-structural genes in the viral RNA 2. *J. Gen. Virol. 78,* 465–467.

6. Koenig, R., Lesemann, D. E., Pfeilstetter, E., Winter, S., Pleij, C. W. A. (2011) Deletions and recombinations with the RNA1 3' ends of different tobraviruses have created a multitude of tobacco rattle virus TCM-related RNA2 species in *Alstroemeria* and *tulip*. *J. Gen. Virol. 92,* 988–996.
7. Koenig, R., Lesemann, D. E., Pleij, C. W. A. (2012) Tobacco rattle virus genome alterations in the *Hosta* hybrid 'Green Fountain' and other plants: reassortments, recombinations and deletions. *Arch. Virol. 157,* 2005–2008.
8. MacFarlane, S. A. (1999) Molecular biology of the tobraviruses. *J. Gen. Virol. 80,* 2799–2807.
9. Park, I. S., Chang, M. U., Kim, K. W., Kyun, H. J. (1998) The viruses in *Gladiolus hybridus* cultivated in Korea 2. Broad bean wilt virus, Cucumber mosaic virus and Tobacco rattle virus. *Plant Pathol. J. 14,* 83–91.
10. Shin, H. Y., Koo, B. J., Kang, S. G., Chang, M. U., Ryu, K. H. (2002) Characterization of Tobacco rattle virus (TRV-K) isolated in Korea. *Res. Plant Dis. 8,* 207–214.
11. Sudarshana, M. R., Berger, P. H. (1998) Nucleotide sequence of both genomic RNAs of a North American tobacco rattle virus isolate. *Arch. Virol. 143,* 1535–1544.
12. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol. 30,* 2725–2729.
13. Visser, P. B., Bol, J. F. (1999) Nonstructural proteins of Tobacco rattle virus which have a role in nematode-transmission: Expression pattern and interaction with viral coat protein. *J. Gen. Virol. 80*, 3273–3280.
14. Yin, Z., Pawelkowicz, M., Michalak, K., Chrzanowska, M., Zimnoch-Guzowska, E. (2014) The genomic RNA1 and RNA2 sequences of the Tobacco rattle virus isolates found in Polish potato fields. *Virus Res. 185,* 110–113.