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Cloning and Phylogenetic Characterization of Coat Protein Genes of Two Isolates of *Apple mosaic virus* from 'Fuji' Apple

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Apple mosaic virus (ApMV), a member of the genus *Ilarvirus*, was detected and isolated from diseased 'Fuji' apple (*Malus domestica*) in Korea. The coat protein (CP) genes of two ApMV strains, denoted as ApMV-K1 and ApMV-K2, were amplified by using the reverse transcription and polymerase chain reaction (RT-PCR) and were analyzed thereafter. The objectives were to define the molecular variability of genomic information of ApMV found in Korea and to develop virus-derived resistant gene source for making virus-resistant transgenic apple. RT-PCR amplicons for the ApMVs were cloned and their nucleotide sequences were determined. The CPs of ApMV-K1 and ApMV-K2 consisted of 222 and 232 amino acid residues, respectively. The identities of the CPs of the two Korean ApMVs were 93.1% and 85.6% at the nucleotide and amino acid sequences, respectively. The CP of ApMV-K1 showed 46.1-100% and 43.2-100% identities to eight different ApMV strains at the nucleotide and amino acid levels, respectively. When ApMV-PV32 strain was not included in the analysis, ApMV strains shared over 83.0% and 78.6% homologies at the nucleotide and amino acid levels, respectively. ApMV strains showed heterogeneity in CP size and sequence variability. Most of the amino acid residue differences were located at the N-termini of the strains of ApMV, whereas, the middle regions and C-termini were remarkably conserved. The ApMVs were 17.0-54.5% identical with three other species of the genus *Ilarvirus*. ApMV strains can be classified into three subgroups (subgroups I, II, and III) based on the phylogenetic analysis of CP gene in both nucleotide and amino acid levels. Interestingly, all the strains of subgroup I were isolated from apple plants, while the strains of subgroups II and III were originated from peach, hop, or pear. The results suggest that ApMV strains co-evolved with their host plants, which may have resulted in the CP heterogeneity.

***Keywords*:** ApMV, apple, coat protein heterogeneity, Fuji cultivar, *Ilarvirus*, phylogeny, variability.

Apple mosaic virus (ApMV) is a species of the genus *Ilarvirus* in the family *Bormoviridae.* The genus *Ilarvirus* comprises a large group of plant viruses, and woody trees are their primary natural hosts (Van Regenmortel et al., 2000; Vaskova et al., 2000; Zimmerman and Scott, 2001). ApMV, *Prunus necrotic ringspot virus* (PNRSV), *Tulane apple mosaic virus* (TApMV), *Prune dwarf virus*, *Lilac ring mottle virus*, and *American plum line pattern virus* (APLPV) are some of the members of the genus *Ilarvirus* (Bachman et al., 1994; Guo et al., 1995; Hammond and Crosslin, 1995; Rybicki, 1995; Sanchez-Navarro and Pallas, 1994; Scott and Ge, 1995). A recent report of the International Committee on Taxonomy of Viruses suggests that members of the genus *Ilarvirus* can be classified into seven major subgroups (subgroups I-VII) based on their antigenic (serological) relationships (van Regenmortel et al., 2000). ApMV belongs to subgroup III, together with PNRSV, *Blueberry shock virus*, and *Humulus japonicus latent virus*.

Apple is one of the natural hosts of ApMV. The virus has a wide host range experimentally and naturally (Fulton, 1972). It has infected over 65 species including apricot, almond, pear, and other woody plants (Fulton, 1972; Petrzik and Lenz, 2002). There is no known vector for members of the genus. The virus is transmitted from infected trees to healthy ones by vegetative propagation and by mechanical inoculation (Fulton, 1972).

Ilarvirus has three genomic RNAs namely RNA1, RNA2, and RNA3 (Bol, 1999), and these viral genomic RNAs are encapsidated in the virus particle by coat protein

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(CP). RNA1 and RNA2 encode viral replicases responsible for virus replication in its host cells (Shiel and Berger, 2000). RNA3 is bicistronic and encodes movement protein (MP) and CP (Shiel et al., 1995). MP plays a role in cell-to-cell movement function and is directly translated from RNA3, whereas, CP is translated from the subgenomic mRNA (RNA4), derived from RNA3 (Alrefai et al., 1994). The CP of *Ilarviruses* forms the shell for the three genome components, and it also plays a major role in the multiple step of replication and initiation, as well as, in the propagation of infection (Ansel-McKinney and Gehrke, 1998; Bol, 1999).

Apple (*Malus* x *domestica* Borkh.) is one of the most widely grown fruit crops in the world. There are a number of several commercial cultivars of apple worldwide. 'Fuji' is the best commercial apple cultivar in Korea. However, poor coloration and yield reduction caused by virus diseases have posed serious problems. Several viruses such as *Apple chlorotic leaf spot virus* (ACLV, *Trichovirus*), *Apple stem pitting virus* (ASPV, *Foveavirus*), ApMV, TapMV, and *Apple stem grooving virus* (ASGV, *Capillovirus*) which infect various apple cultivars have been reported in the world (Germa-Retana et al., 1997; Martelli and Jelkmann, 1998; Petrzik and Lenz, 2002; Shiel et al., 1995). Significant yield reduction of apple up to 60% is possible, especially for frequent mixed infections (Petrzik and Lenz, 2002). However, little is known on comparative sequence and pathological information, as well as, the general characterization of these apple-infecting viruses. These can interfere in the molecular breeding program for virus-resistant transgenic apple in Korea.

In this study, molecular characterization of ApMV from 'Fuji' apple cultivar in Korea especially for the CP gene was carried out as the first step for molecular breeding strategy. A comparative analysis of CP gene of other ApMV strains was also described in this study.

Materials and Methods

Virus sources and survey of virus incidence. ApMV was isolated from diseased symptomatic apple trees (*Malus domestica* L.) var. 'Fuji' (Fig. 1) in the experimental fields of the National Horticultural Research Institute, Suwon, Korea. Two isolates of ApMV from diseased leaves of 'Fuji' apple, designated as ApMV-K1 and ApMV-K2, were used as sources of virus. ApMV-K1 was obtained from the Plant Virus GenBank (Seoul, Korea; http://www.virusbank.org). These viruses were mechanically inoculated on cotyledons of cucumber (*Cucumis sativus* L. cv. Lemon; Lake Valley Seed, Inc, USA) for propagation of virus as previously described (Alrefai et al., 1994). The virus was detected from two varieties (Fuji and Golden Delicious) of apple, and symptom expressions were compared.

Fig. 1. Yellow leaf spots and mosaic leaf symptoms of ApMV in 'Fuji' apple cultivated in Korea (Left: healthy; others: ApMV-infected leaves).

Purification of virus and RNA preparation. Virus particles were purified from systemically infected cucumber leaves by extraction and clarification with chloroform, followed by differential centrifugation and sucrose density-gradient method (Alrefai et al., 1994). Total genomic RNAs of the virus were extracted from purified virus preparations by SDS/proteinase K-phenol extraction method as described by Ryu and Choi (2002).

Reverse transcription and polymerase chain reaction (RT-PCR) for amplification of the ApMV CP gene. Two ApMV-specific primers were designed based on multiple alignments of nucleotide sequences of coat protein genes of eight known ApMV strains retrieved from the GenBank database. PAPCP3-1 (5'-CTAACAAATCTTCATCGATAAG-3') and PAPCP5 (5'-TCTAACATGGTCTGCAAGTAC-3') were reverse and forward primers, respectively. The primers were obtained from the Plant Virus GenBank (Seoul, Korea) and used for amplification of CP gene of ApMV. RT reaction was performed in 20 μl reaction volume containing 4 μl of 5x reaction buffer (Invitrogen), 1.25 mM each dNTP, 35 U ribonuclease inhibitor (MBI), 20 pmoles primer, and 0.5 U AMV reverse transcriptase (Invitrogen). Reaction mixture was overlaid with mineral oil (Sigma) and incubated at 45°C for 1 h (Jung et al., 2000). PCR was carried out in a final volume of 50 μl containing 5 μl 10 x PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 μl 50 mM $MgCl_2$, 1 μl 10 mM dNTPs, 0.4 μl *Taq* DNA polymerase (5 U/μl) (Invitrogen), 2 μl cDNA reaction mixture from the RT, and 10 pmole of PAPCP3-1 and PAPCP5 primers. PCR cycle was performed in a PTC 100 thermocycler (MJ Research) or a i-cycler (Biorad) followed by the thermal cycling scheme: 2 minutes at 94 °C, 35 cycles for 30 seconds at 94°C, 30 seconds at 56°C, and 90 seconds at 72°C; and a final extension for 10 minutes at 72°C (Yoon et al., 2002). The amplicons of target DNA were examined on agarose gel electrophoresis.

cDNA cloning and sequence determination. The amplified RT-PCR product of about 700 bp was purified by the Qiaquick PCR purification kit (QIAGEN). Purified RT-PCR products were ligated with pGEM-T-Easy vector (Promega Corp.) by TA

Table 1. Virus name, original host, sequence accession numbers and their characters used in this study

Virus	Original host	Coat protein[a]	ORFs size[b]	GenBank accession No.	References
ApMV-K1	*Malus domestica* cv. Fuji	669	222	AF548367	In this work
ApMV-K2	*Malus domestica* cv. Fuji	699	232	AY125977	In this work
ApMV-ApH	*Malus* spp.	672	223	AY054385	Petzik and Lenz (2002)
ApMV-Pru	*Prunus domestica* (prune)	657	218	AY054386	Petzik and Lenz (2002)
ApMV-Hop	*Humulus lupulus*	657	218	AY054387	Petzik and Lenz (2002)
ApMV-Alm	*Prunus dulcis* (Mill.)Webb	663	220	AY054388	Petzik and Lenz (2002)
ApMV-Pear	*Pyrus* ssp. (pear)	657	218	AY054389	Petzik and Lenz (2002)
ApMV-PV32	*Malus* spp.	681	226	U03857	Sanchez-Navarro and Pallas (1997)
ApMV-U1	*Malus* spp.	666	221	L03726	Alrefai et al. (1994)
ApMV-F	*Malus domestica*	669	222	U15608	Shiel et al. (1995)
TApMV	*Malus* spp.	639	212	AF226162	Scott et al. (1995)
APLPV	*Prunus americana*	651	216	AF235166	Scott and Zimmerman (2001)
PNRSV	*Prunus persica*	675	224	L38823	Hammond and Crosslin (1995)

[a] Number of nucleotides.
[b] Number of amino acid of cp.

cloning procedure according to the manufacturers instruction. They were transformed into *Escherichia coli* strain JM109 competent cells. Recombinant plasmids harboring cDNA insert were selected from transformants and used for nucleotide sequencing. These were sequenced with T7 promoter and M13 reverse universal primers by the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) on an automated DNA ABI PRISM 310 sequencer (PE Applied Biosystems).

Sequence comparison and phylogenetic tree analysis. Nucleotide sequences and deduced amino acid sequences of CP of ApMV strains and some members of the genus *Ilarvirus* were aligned using the DNASTAR software package (Madison, WI). Sequences of eight different strains of ApMV and three *Ilarviruses* for analysis were retrieved from the GenBank database as listed in Table 1, and were analyzed with that of the two Korean ApMV strains. Phylogenetic tree analysis was performed using a DNAMAN package (version 5.1, Lynnon Biosoft, Canada).

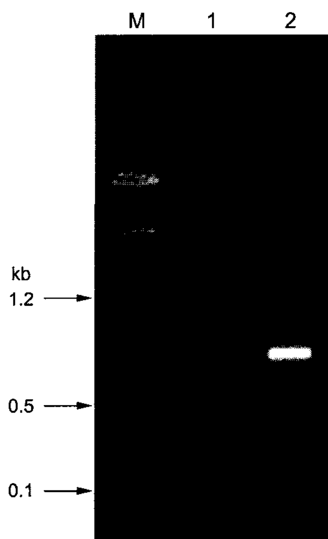

Fig. 2. Agarose gel electrophoresis of RT-PCR products of ApMV coat protein gene by using PAPCP3-1 and PAPCP5 primers. Lane M: 1kb ladder (MBI Co.); Lane 1: healthy control; Lane 2: ApMV-infected leaves (ApMV-K2).

Results and Discussion

Symptoms of ApMV-infected apple trees. ApMV caused different leaf symptoms depending on the apple cultivar and the season. Systemic distinct yellow leaf spots were observed in between leaf veins of 'Fuji' apple (Fig. 1), whereas, veins showed necrosis in 'Golden Delicious' apple (data not shown). These symptoms appeared clearly

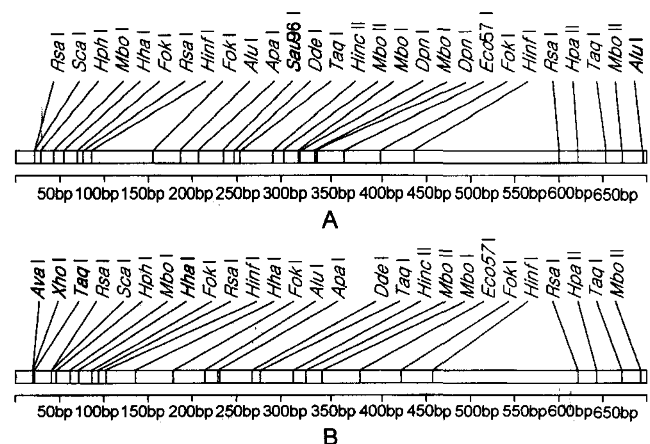

Fig. 3. Linear illustration of restriction endonucleases recognition sites found in two strains of ApMV coat protein genes. Panel A; ApMV-K1; Panel B: ApMV-K2.

Fig. 4. Multiple alignments of strains of ApMV and other *Ilarviruses* based on amino acid sequences of CP genes. Amino acid residues identical to ApMV-K1 are marked with asterisk (*), and gaps with hyphen/dash (–). Open box represents variable regions of ApMV strains.

in late spring to early summer and then diffused to mild symptoms in Korea. Four apple-infecting viruses, ACLSV, ASGV, ASPV and ApMV, have been reported in Korea, and their infection rate in 1993-2001 was 0.4-57.1% (National Horticultural Research Institute, Korea, unpublished results). ApMV caused mosaic symptom in apple, line-pattern in plum tree, and mosaic in rose (Fulton, 1972). There is much variation in symptoms with different isolates of ApMV (Fulton, 1972). Petrzik and Lenz (2002) recently suggested that the host could influence the observed variability along the type of strains of ApMV. As observed by authors of this study, as well as, other researchers, host (cultivars) and virus (strains) interaction may contribute to the pathology, especially symptom expression pattern, of ApMV.

Amplification and cloning of CP genes of two isolates of ApMV. To define the molecular variability of genomic information of ApMV found in Korea and to get virus-derived resistant gene source for developing virus-resistant transgenic apple, coat protein (CP) genes of two ApMV strains (ApMV-K1 and -K2) were amplified by using the RT-PCR and where analyzed thereafter. ApMV-specific primers (see materials and methods) used in this study successfully amplified single 700 bp PCR products, which covered full-length CP gene of the two ApMVs, as shown in Figure 2. The PCR amplicons for the ApMVs were cloned and used for sequence determination.

Sequence analysis of CP of two Korean isolates of ApMV. The CPs of ApMV-K1 and ApMV-K2 consisted of 222 and 232 amino acid residues, respectively. Sequences of CP gene for ApMV-K1 and ApMV-K2 determined in this study appeared in the GenBank database under accession numbers AF548367 and AY125977, respectively. To identify the two Korean ApMV strains, 25 restriction enzymes were analyzed for the amplified ApMV CPs: *Alu*I, *Apa*I, *Ava*I, *Ava*II, *Ban*II, *Bsp*6II, *Cla*I, *Dde*I, *Dpn*I, *Eco*57V, *Fok*I, *Hae*III, *Hha*I, *Hinc*II, *Hinf*I, *Hpa*II, *Hph*I, *Mbo*I, *Mbo*II, *Rsa*I, *Sau*96I, *Sca*I, *Taq*I, *Xho*I, and *Xmn*I. Restriction analysis revealed that the CP of ApMV-K1 had 45 recognition sites by 23 enzymes (Fig. 3A), whereas, 47 sites by 25 enzymes were found in ApMV-K2 with cutting sites of *Ava*I, *Xho*I and *Taq*I, which were not found in ApMV-K1 (Fig. 3B).

Identities of CPs of two Korean ApMVs were 93.1% and 85.6% at the nucleotide and amino acid sequences, respectively. ApMV-K1 showed 46.1-100% and 43.2-100% identities to the eight different ApMV strains at the nucleotide and amino acid levels, respectively. Interestingly, the CP of ApMV-K1 was identical with that of ApMV-F, which was also isolated from cultivated apple in USA. The CP of ApMV-K2 consisting of 232 amino acid residues was the longest among the strains of ApMV so far, and the N-terminal nine amino acid residues found only in ApMV-K2 contributed to the length (Fig. 4). In this study, these extra sequences found only in ApMV-K2 were not excluded from the PCR artifact. These sequences did not affect the open reading frame of its CP. When comparing CPs of strains of ApMV, ApMV-PV32 (an isolate from *Malus* spp.) only showed less than 50% amino acid similarity with all the known ApMV strains, and 85.7% similarity with that of PNRSV, indicating it is a strain of PNRSV. Hence, ApMV-PV-32 should be reclassified as a strain of PNRSV based on the sequence analysis. ApMV strains except PV32 share over 83% and 78% homologies at the nucleotide and amino acid levels, respectively (Table 2). ApMV strains showed heterogeneity

Table 2. Percent of similarity of ApMV coat protein gene with other three species of the genus *Ilarvirus*, using CLUSTAL method

Nucleotide / Protein	1[a]	2	3	4	5	6	7	8	9	10	11	12	13
1. ApMV-K1		93.1	90.2	84.3	84.9	83.0	84.8	46.1	93.8	100.0	26.9	25.2	45.9
2. ApMV-K2	85.6		94.8	87.5	88.1	86.3	88.0	46.6	92.7	95.5	26.3	26.4	48.4
3. ApMV-ApH	82.0	95.5		87.4	88.4	86.7	88.6	49.6	90.3	90.4	25.2	25.3	50.4
4. ApMV-Pru	78.9	90.4	90.8		97.7	86.6	97.3	47.0	84.3	84.3	24.9	27.2	48.6
5. ApMV-Hop	79.4	90.8	91.3	97.2		87.5	98.9	44.6	84.9	84.9	25.5	27.3	49.5
6. ApMV-Alm	78.6	90.5	89.1	88.5	89.0		87.2	44.9	83.3	83.0	26.1	26.6	50.1
7. ApMV-Pear	79.4	90.8	91.3	96.8	98.2	89.0		44.3	84.8	84.8	25.5	28.0	49.0
8. ApMV-PV32	43.2	48.2	49.3	47.7	49.1	50.0	48.2		40.2	46.9	22.8	26.1	86.2
9. ApMV-U1	82.8	91.4	86.9	83.9	84.4	83.2	84.4	45.7		93.6	26.8	26.1	45.2
10. ApMV-F	100.0	85.6	82.0	78.9	79.4	78.6	79.4	43.2	82.8		26.9	25.2	46.3
11. TApMV	17.0	17.9	17.5	18.4	17.9	18.4	18.4	13.7	17.9	17.0		25.0	21.0
12. APLPV	20.4	21.3	20.8	19.9	19.9	20.8	20.4	22.2	23.1	20.4	17.5		25.5
13. PNRSV	46.8	52.2	53.4	51.8	53.2	54.5	52.3	85.7	48.9	46.8	15.6	21.3	

[a] Virus names are the same of the row.

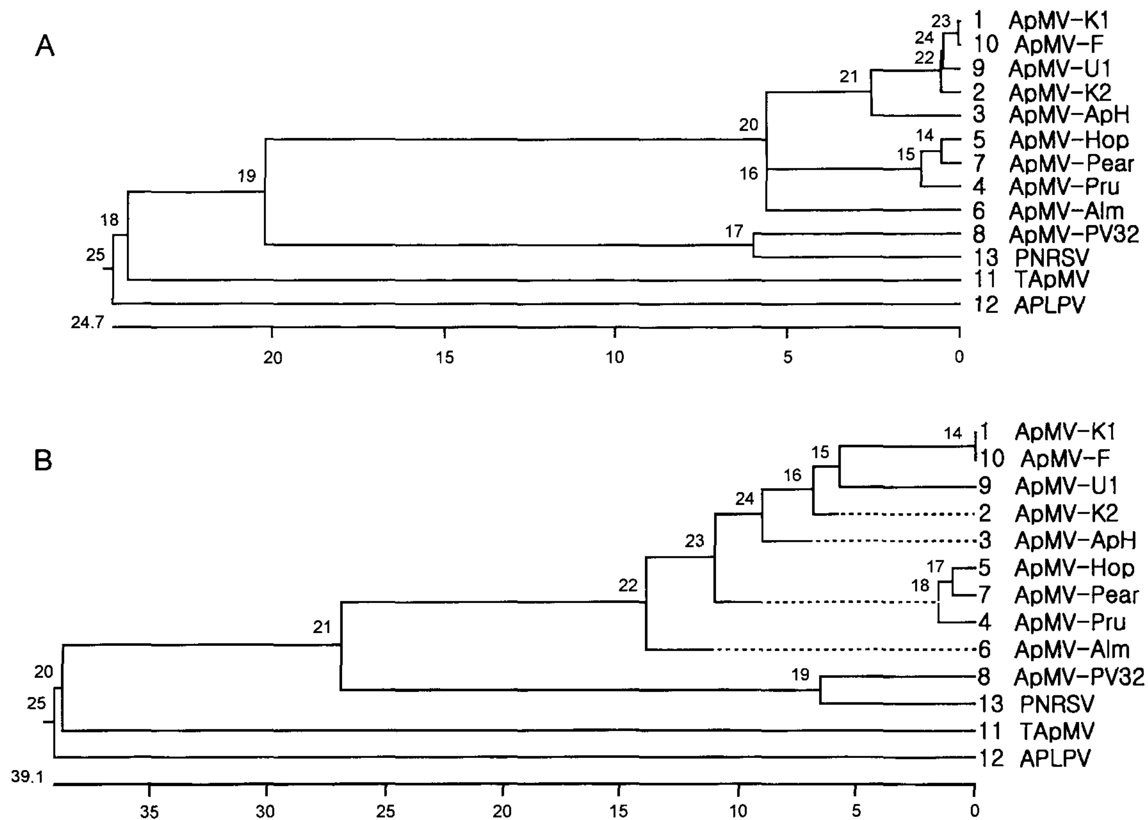

Fig. 5. Phylogenetic trees of strains of ApMV with other selected *Ilarviruses* using CLUSTAL method with residue weight table based on nucleotide (**A**) and amino acid (**B**) sequences of CP genes.

in CP size (218-232 amino acids) and sequence variability (Fig. 4, Table 1). Some deletions of amino acids were found in the 65th to 76th residues in strains of ApMV, and these contributed to the heterogeneity of CP lengths (Fig. 4). Amino acid residue difference was found in the N-termini of the strains of ApMV, while the middle and C-termini regions were remarkably conserved (Fig. 4). ApMVs were 17.0% (with APLPV) to 54.5% identical (with PNRSV) with three other species of the genus *Ilarvirus* (Table 2).

Phylogenetic analysis of ApMVs and other members of the genus *Ilarvirus*. To determine the relationship among strains of ApMV and other *Ilarviruses*, phylogenetic tree analysis was performed using the Clustal algorithm. All strains of ApMV can be classified into three subgroups (I, II, and III) based on the phylogenetic analysis of CP gene in both nucleotide and amino acid levels: subgroup I (ApMV-K1, -K2, -F, -ApH and -U1); subgroup II with ApMV-Hop, -Pear and Pru; and subgroup III with ApMV-Alm (Fig. 5A and 5B). Interestingly, all the ApMV strains of subgroup I originated from apple plants, and strains of subgroups II and III were isolated from peach, hop, or pear. The results suggest that ApMV strains co-evolve with their host plants, and that this may result in CP heterogeneity.

Petrzik and Lenz (2002) analyzed more than 50 plants for the presence of ApMV (mostly isolated from apple trees). They found that 4 out of 11 isolates of ApMV possess insertions of various lengths of CPs. Two pathotypes and three serotypes were found to be closely related to PNRSV isolates, which were antigenically related and clustered together with ApMV in the same subgroup (Mink et al., 1987). Aparicio and Pallas (2002) recently reported the heterogeneity of CPs of 15 isolates of PNRSV.

In conclusion, ApMV-K1 and ApMV-K2 were strongly related with other ApMV strains. This report provides information on the composition of ApMV CP gene. Production of virus-resistant apple plants is now in progress.

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