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Characteristics of *Potato virus Y* Isolated from Paprika in Korea

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A virus isolate collected from infected paprika (*Capsicum annuum* var. grossum) was characterized as *Potato virus Y* (PVY) based on biological, serological, cytopathological, and molecular properties. In host range studies, the paprika isolate produced the mosaic symptom on some tobacco, tomato and pepper (*Capsicum annuum*). A new paprika isolate also infected potato cultivars which is different biological characteristic compared to the other popular potyvirus infecting paprika, *Pepper mottle virus* (PepMoV). Previously reported PVY strains, PVY^O and PVY^N did not infect pepper and typical PepMoV isolates did not infect potato. Distinctive inclusion patterns of the scroll, pinwheel, long laminated inclusions, and helper components in the cytoplasm of infected cells were also different to those observed by the typical PVY isolate infections. However, the paprika isolate reacted to the monoclonal antibody of PVY^N strain with high absorbance readings. RT-PCR amplification, cloning, and sequencing of the 3' untranslated region and a part of coat protein gene also added additional evidence of the paprika isolate as the PVY^N-related isolate. Multiple alignments as well as cluster dendrograms of PVY-paprika isolate revealed close phylogenetic relationship to the PVY^N subgroup. Altogether, these results suggest that a new PVY isolate infecting paprika contained distinct characteristics compared to the other previously described PVY strains with closer relationship to the PVY^N strain.

Keywords : characterization, paprika, pathogenesis, PVY^N

Potato virus Y (PVY) is the type member of the genus *Potyvirus* of the family *Potyviridae* (Francki et al., 1991), which is the largest viral group and contains many economically important viruses. The virus has a long filamentous particle containing one single-stranded, positive sense RNA genome of approximately 9.7 kb. PVY is an important pathogen infecting potato, pepper, tobacco, tomato, and other solanaceous plant species causing significant yield losses worldwide (DeBokx and Huttinga, 1981) and is naturally transmitted by many aphid species in a non-persistent manner.

PVY isolates have been differentially classified into three distinct groups depending on the reactions that they produce in different potato (*Solanum tuberosum*) cultivars and in the indicator plants *Physalis floridana* and *Nicotiana tabacum* L. cv. Samsun and White Burly (DeBokx and Huttinga, 1981). The PVY^O group (common strains) causes mottling in tobacco and crinkling, rugosity or streaks in potato. The PVY^N group (tobacco veinal necrosis strains) induces necrotic symptoms in tobacco and mild mottling in potato cultivars. The PVY^C group (stipple streak strains, including potato virus C) cause mosaic or stipple streak in potato cultivars (De Bokx and Huttinga, 1981). However, not all strains fit into these three groups. PVY isolates infecting pepper are less studied and classified as PVY-0, PVY-1, and PVY-1-2 in accordance with their ability to overcome resistance genes. Within these groups, isolates are further defined as "common" or "necrotic" type. Common isolates induce vein-banding symptoms in pepper, while necrotic ones also cause vein necrosis.

Biological and serological comparisons of PVY from pepper and PVY from potato or tobacco suggest that PVY from pepper belongs to a different PVY group. The taxonomy of potyviruses based on biological and serological properties is unsatisfactory and several authors hypothesized that within the potyvirus group there is a continuum of variants or strains that cannot be distinguished into strains and species. In addition to the biological and serological data, nucleotide sequence identities of coat protein (CP) or 3'-untranslated region (UTR) have been increasingly used to differentiate distinct species from strains (Frankel et al., 1989; Shukla and Ward, 1989). Further, van der Vlugt et al. (1993) reported the subgrouping of PVY isolates based on the CP and 3'-UTR sequence identities that matched groupings based on biological and

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serological criteria.

Virus diseases causing yield loss and reducing the quality of paprika (*Capsicum annuum* var. grossum) were surveyed in 21 fields of Korea. There was an average disease incidence of 1.47%, but infection rate and kind of viruses were different upon the paprika cultivating provinces. PVY, *Pepper mottle virus* (PepMoV), *Pepper mild mosaic virus* (PMMoV), *Cucumber mosaic virus* (CMV), *Broad bean wilt virus* (BBWV) and some other unknown viruses were identified from paprika plants showing various symptoms (Song et al., 2002). Occurrences of virus diseases on paprika were surveyed in Jeonnam province from 1999 to 2003 and the collected samples showing virus-like symptoms were tested using ELISA. Disease incidences of virus disease were 4.5%, 17.5%, and 4.9% in 2000, 2002, and 2003, respectively. As the results of investigation of the seasonal incidence with the growing stages of plant, virus symptom was not observed at seedling stage and was slightly increased from the planting time to the first harvesting time, but was dramatically increased at the second harvesting time. Virus diseases were more severe on the vinyl house cultivation than on the green house cultivation. A total of 313 samples showing virus-like symptoms were collected and analyzed. Both indicator host species and DAC-ELISA revealed that PepMoV was detected in 30-70%, BBWV in 10-15% and CMV in 2-10% during surveyed years described previously (Ko et al., 2002).

In this study, we isolated PVY isolate from paprika and compared its characteristics to the other PVY strains, using biological, serological, and cytological properties. We also sequenced 3'-UTR and a part of CP-gene nucleotide sequence and analyzed phylogentic relationship to the other PVY strains.

Table 1. Symptoms developed on indicator plants inoculated with PVY-paprika isolate[a]

Indicator plant test	PVY-paprika	PVY-pot-Chw
Chenopodium amaranticolor	cl/–	–/–(cl/–)
C. quinoa	cl/–	nl/–
Nicotiana benthamiana	cl/M	cl/cl, sM
N. tabacum 'bright yellow'	cl/M	cl/vc, mM
N. tabacum 'X-nc'	cl/M	cl/vc, mM
N. tabacum 'Samsun"	cl/M	cl/vc, mM
Physalis floridana	cl/sM	cl/cl, M
Petunia spp.	cl/M	cl/M
Tetragonia expansa	cl/–	cl/–
Datura stramonium	–/–	–/–
Sesamum indicum L.	–/–	–/–
Perilla frutescens	–/–	–/–
Impatiens balsamina L	–/–	–/–
Zinnia elegans Jacq.	–/–	–/–
Cucumis sativus L.	–/–	–/–
Cucumis melo L.	–/–	–/–
Citrullus lanatus	–/–	–/–
Cucurbita moschata Duch	–/–	–/–
Raphanus sativus L.	–/–	–/–
Brassica campestris	–/–	–/–
Brassica rapa L.	–/–	–/–
Chrysanthemum coronarium	–/–	–/–
Phaseolus vulgaris L.	–/–	–/–
Vicia faba	–/–	–/–
Glycine max Merr.	–/–	–/–
Phaseolus angularis	-/-	-/-
Phaseolus radiatus L.	-/-	-/-
Vigna sinensis King	-/-	-/-

[a]M, mosaic; mM, mild mosaic; vc, vein clearing; cl, chlorotic local lesion; nl, necrotic local lesion; –, no symptom; Inoculated leaf/ Upper leaf.

Materials and Methods

Sample collection. During October of 1999 to March 2000, a total of 121 samples showing virus-like symptoms were collected in main paprika growing provinces of Korea including Gyeonggi-do, Gyeongsangnam-do, Jeollanam-do and Jeollabuk-do (Table 1). Disease incidence was calculated as the number of plants showing virus-like symptoms relative to the total number of plants observed in each area.

PVY isolates and host range studies. Although we did not observe any distinct differences of symptoms, two isolates showed vein banding. Each isolate was propagated in *Nicotiana tabacum* cv. 'X-nc' through three successive sap inoculations under insect proof glass house conditions. To determine the infectivity of virus isolates and the symptoms induced on the test plant, 5-10 plant seedlings of each of the species, *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana benthamiana*, *N. tabacum* cv. 'bright yellow', *N. tabacum* cv. 'X-nc', *N. tabacum* cv. 'Samsun', *Physalis floridana*, *Petunia* spp. at the 3-5 leaf stage were inoculated by sap inoculation in 0.1 M phosphate buffer, pH 7.0. The plants were put in an insect-free glasshouse maintained at 20-25°C with 12 h light period. Disease symptoms were recorded thrice weekly for 30 days. Both symptomatic and non-symptomatic plants were verified for PVY infection by DAC-ELISA and by electron microscopy.

DAC-ELISA. Because polyclonal antibodies can not differentiate PVY strains due to the high level of epitope conservation on the CPs (Shukla & Ward, 1989), a PVY specific monoclonal antibodies were used. Direct-antibody coated enzyme linked immunosorbent assay (DAC-ELISA) was conducted essentially as described by Clark & Bar-

Joseph (1984) using monoclonal antibodies (MAb) purchased from Adgen (United kingdom). The MAbs and conjugate were both diluted 1:1,000 and all incubations were carried out at 37°C for 2 h except for the substrate which was incubated for 30 min. Quantitative measures of generated *p*-nitrophenol were made by determining absorbance at 405 nm (A_{405}) in an EL312e EIA model spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). Twice absorbance value was considered positive to negative control.

Electron microscopy. Two weeks after inoculation, infected leaves were harvested and immediately cut with a sharp blade (which was washed with 75% alcohol after every cut) into 1-3 mm thick pieces. The newly cut pieces were immediately fixed with 2.5% glutaraldehyde in Millonig's phosphate buffer, pH 7.0 at 4°C. The pieces were thoroughly rinsed in Millonig's phosphate buffer before being fixed with 2% osmium tetraoxide for 90 min. The pieces were then stained overnight in 1.0% uranyl acetate at 4°C and rinsed in distilled water. The pieces were dehydrated six times each with 50-100% ethyl alcohol for 50 min. The dehydrated pieces were embedded in spur resin and hardened overnight at 60°C. Ultra-thin sections of 80 nm thickness were sliced using ultramicrotome and a knife. The sections were then stained twice, with 2% uranyl acetate for 20 min then with 0.5% lead citrate for 10 min. For interpretation of results, the sections were observed under electron microscope LEO 912AB at 80 kV.

RT-PCR, cloning and sequencing. Total RNAs were extracted from infected leaf samples essentially as described by Prescott and Martin (1987). The 3'-terminal region of paprika isolate of PVY comprising part of the CP gene and the 3'-UTR was amplified following the RT-PCR procedure described by Pappu et al. (1993). The amplified PCR products were cloned in pGEM-T vector (Promega) and sequenced. PVY-paprika sequences obtained were phylogenetically compared to those of other PVY (GenBank and EMBL) using the multiple sequence alignment application of DNAMAN version 4.0 (Lynnon Biosoft, Quebec, Canada) full optimal sequence alignments and neighbor-joining method options of Saitou and Nei (1987) with 1000 bootstrap (Felsenstein, 1985) replications. Percent nucleotide (nt) and ORF amino acid (aa) sequence identities between virus isolates were calculated using the distance between all pairs of sequences in the multiple alignments (Altschul et al., 1997). PVY (CP-UTR) sequences used for comparison and their database accession numbers were as follows: AJ223592 (PVY-N854 isolate), AJ390285 (PVY-N-RB isolate), AJ390295 (PVYN-S-NTN isolate), D12570 (necrotic-PVY-T isolate), X97895 (N605 isolate), Z70237 (Nysa isolate), U09508 (N27-92 isolate), AJ223593 (O768 isolate), D12539 (PVY-O isolate), U09509 (PVYO-Canadian isolate), AJ390289 (v942490 isolate), U10378 (Hungary pepper isolate), M22470 (N-PVY isolate), and M95491 (Hungarian isolate).

Results

Sample collection. A preliminary survey of viruses was conducted from October 1999 to march 2000, and 121 samples showing virus-like symptoms were collected in the paprika production areas in Korea. In 21 fields, there was an average disease incidence of 1.47%, but infection rate of viruses according to province was different, i.e., 5.25% in Gyeonggi-do, 4.53% in Jeollanam-do, 0.32% in Gyeongsangnam-do and 0.24% in Jeollabuk-do. Of ninety-six isolates tested; 3 of PVY, 42 of PepMoV, 19 of PMMoV, 20 of CMV, 6 of BBWV, 4 of mix-infection of CMV plus PepMoV and 2 of unknown viruses were identifed from samples of 21 areas.

Table 2. Infectivity of PVY-paprika and PVY^{o} isolates in tomato, red pepper and potato cultivars

Indicator plant test		PVY-paprika	PVY^{o}-potato
Lycopersicon esculentum cv.	'Dotaerang'	+[a]	+
	'Dotaerang B'	+	+
	'House Dotaerang'	+	+
	'Yeonggwang'	+	+
	'Seogwang'	+	+
	'Ponterosa'	+	+
	'Yeongmuja'	+	–
	'Mini'	+	–
	'PaePae"	+	–
	'KoKo'	+	–
Capsicum annuum cv.	'Boogwang'	+	–
	'DongBang'	+	–
	'Geosung'	+	–
	'PungCheon'	+	–
	'KumTop'	+	–
	'DaeWang'	+	–
	'DaeMyong'	+	–
	'JoYang'	+	–
	'ChungOk'	+	–
	'UmSung'	+	–
Solanum tuberosum cv.	'Daeji'	+	+
	'Soomi'	+	+
	'Namjack'	+	+
	'Jopyung'	+	+

[a] +, positive; –, negative.

Host range studies. Of 29 indicator plants to sap inoculation with PVY-paprika tested for their susceptibility, only *Solanaceae* species were susceptible. PVY-paprika isolate caused mild mosaic and mosaic symptoms in *N. tabacum* and *S. tuberosum*, but no symptoms were observed on *Sesamum indicum* L. (Table 1). In addition, PVY-paprika isolate infected all varieties of *Lycopersicon esculentum* and *Capsicum annuum* (Table 2).

Serological relationships and electron microscopy. PVY-paprika isolate reacted strongly with the monoclonal antibody to PVY^{N}, but did not reacted with monoclonal antibody to PVY^{O} and $PVY^{N+O/C}$. When PVY-paprika was observed under EM, the typical cytoplasmic inclusions of pinwheels, scrolls, laminated aggregates and helper component in cells of *Nicotiana tabacum* cv. 'X-nc' were observed, while no inclusions of helper component were observed in infected tissues with the other PVY strains (Fig. 1).

Nucleotide sequence of the part of the CP and 3'-UTR gene. The sequenced region included in the 780 bp nucleotides from PVY-paprika isolate using the WCIEN primer. The ORF was followed by the UTR of 326 nucleo-

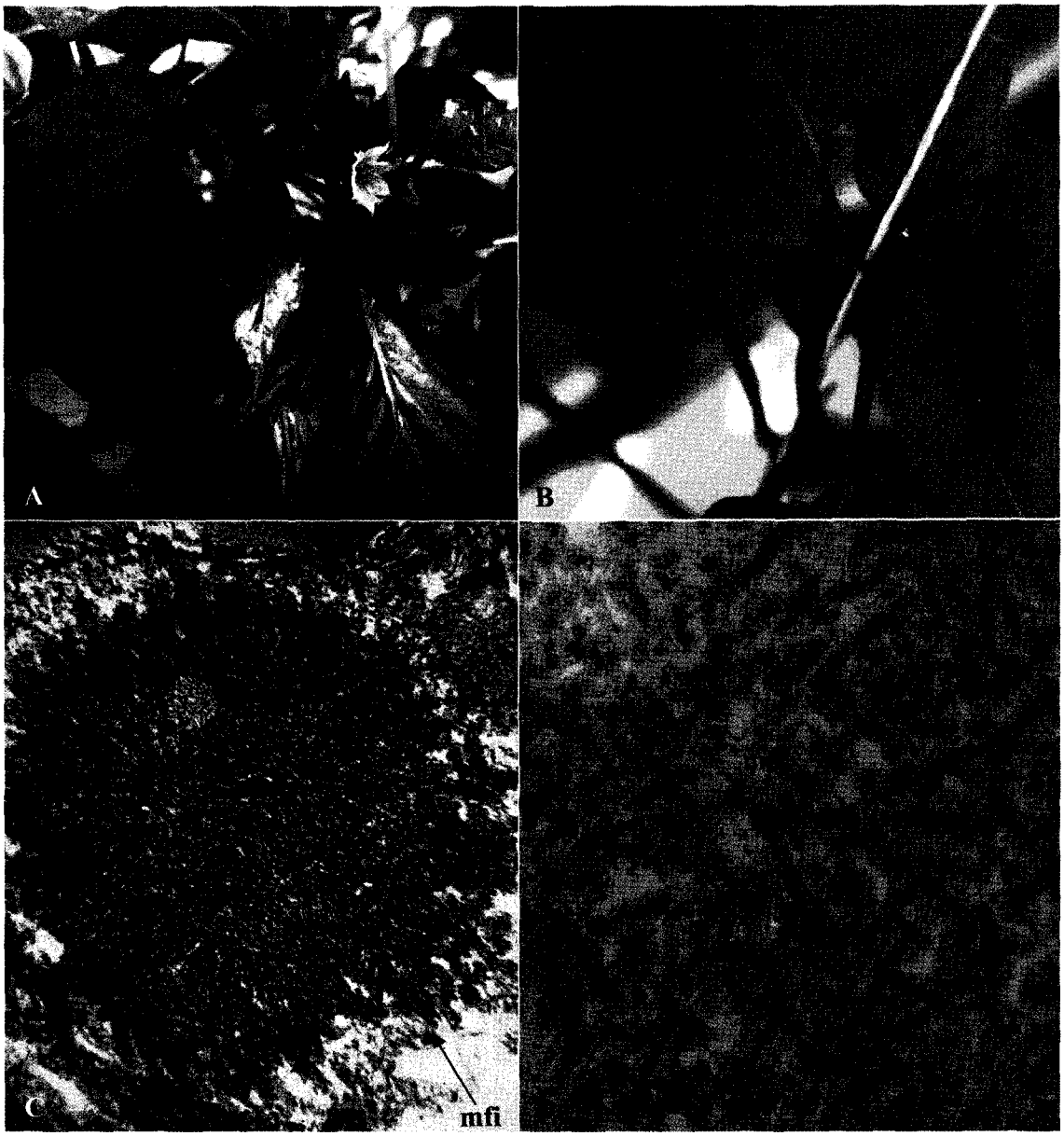

Fig. 1. Paprika plants showing severe mosaic (**A**) and mosaic (**B**) symptoms caused by PVY-paprika isolate in the fields. Electron micrographs (**C** & **D**) showing masses of fibrillar inclusions (mfi) in cells of *Nicotiana benthamiana*.

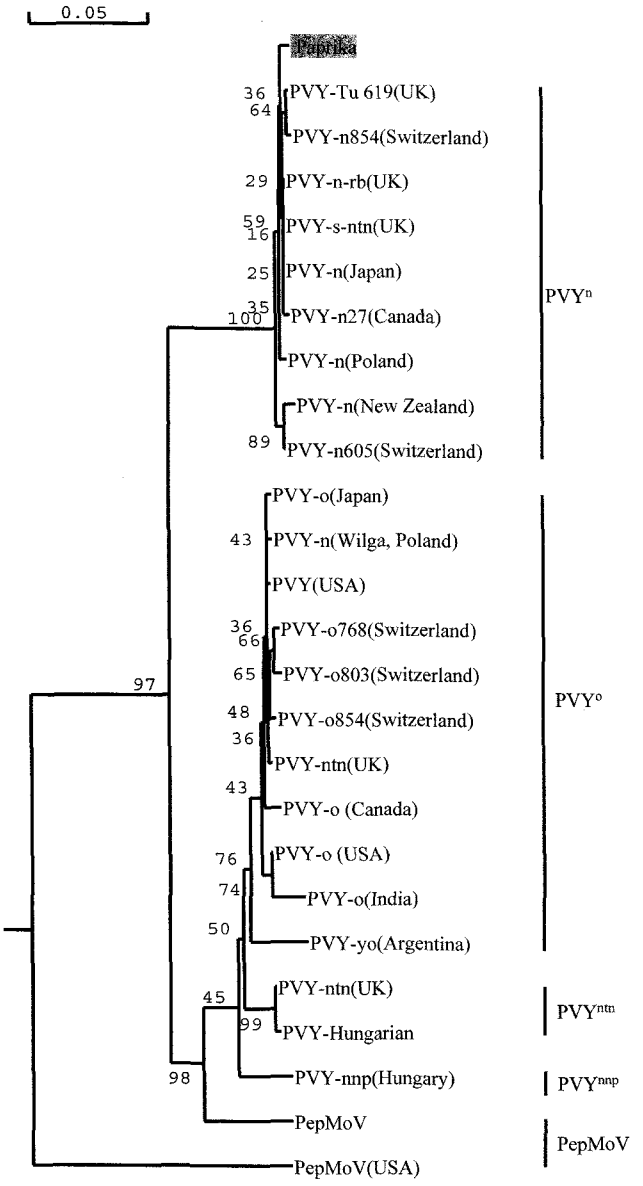

Fig. 2. Phylogenetic tree constructed from nucleotide sequence alignments of the 3'-UTR fragments of PVY-paprika and the other PVY strains and closely related PepMoV isolates.

tides excluding the poly (A) tail. Comparative sequence analysis showed 85.3% identity in the 3'-UTR of potato and paprika isolates. The 3'-UTR sequences of paprika isolate were compared with 24 biologically and geographically distinct PVY isolates. The multiple alignments showed 84-99% identity (data not shown). The cluster dendrogram based on 3'-UTR sequences clearly placed the PVY-paprika isolate within the PVY^N subgroup (Fig. 2).

Discussion

The potyviruses infecting paprikas caused the various symptoms, but there were no clear difference to host plant reactions according to symptom type. Therefore, a PVY-paprika isolate showing vein banding was selected out of 72 PVY isolates obtained from paprika fields in Korea. PVY-paprika isolate was identified on the basis of biological, serological, cytological, and molecular properties. Host range of the virus was restricted to *Solanaceae* (Table 1). PVY-paprika isolate produced symptoms in indicator plants and potato cultivars similar to those caused by the PVY^O group, but could systemically infect pepper and tomato cultivars and therefore could not be considered to belong to any of the previously described PVY^O strain (Park et al., 1984). Cytologically, infected cytoplasms of PVY-paprika isolate accumulated the peculiar inclusion patterns as helper components consistent with those caused by the PepMoV group. In the serological tests, the paprika isolate could be detected using monoclonal PVY^N specific antiserum, but not by the PVY^O.

In comparison with the 3'-UTR and a part of CP-gene were cloned, sequenced, and aligned with the same regions of reported PVY strains. Multiple alignments as well as cluster dendrograms showed that PVY paprika isolate belongs to the PVY^n subgroup. To know the exact identity of these isolates, its 3'-UTR sequences were cloned, sequenced and compared with 24 distinct PVY isolates. High degree of 3'-UTR sequence identities (84-99%) of both potato-Jj and tobacco isolates with known PVY isolates confirmed them as strains of PVY as they were within the cut off range of 83-99% for identifying strains of the same virus (Frankel et al., 1989), whereas 93% identity in Potato-chw. Both potato-Jj and tobacco isolates differed in their 3'-UTR sequences with that of a known tobacco vein necrotic strain of PVY occurring in Korea (PVY-VN) indicate the existence of variation in the necrotic isolates of PVY within Korea (Cheong et al., 1992). The 3'-UTR has been shown to regulate symptom severity (Rodriguez-Cerezo et al., 1991).

Hence further studies involving additional PVY isolates representing different agro-climatic regions of the country are required in order to map the existence of various distinct PVY isolates. This would help in developing an effective strategy for managing the viruses. Biological and serological comparisons of PVY from pepper and PVY from potato or tobacco suggest that PVY from pepper belongs to a different potyviral species (Riechmann et al., 1999). On the basis of serological properties and host plant reactions PepMoV has been classified as a potyvirus related to, but distinct from, the other pepper-infecting potyviruses, PVY and *Tobacco etch virus*. Recent amino acid and nucleotide sequence data show that PepMoV is more closely related to PVY than previously assumed (Bhat et al., 1999). PepMoV shows a high degree of homology to various PVY strains in

both the CP and the 3'-UTR sequences, while unrelated potyviruses are generally less homologous in these regions. Detailed CP amino acid sequence and 3'-UTR nucleotide sequence comparisons described in this paper confirm the close relationship between PepMoV and PVY and it is concluded that the isolate sequenced indeed represents a strain of PVY. Sequence data for several strains of PVY gave two groups with closer relationships among strains in a group than between groups. To further clarify phylogenetic relationship between PVY-paprika and the other PVY strains, we are currently conducting full genome analysis of PVY isolates obtained from potato, tobacco, pepper, paprika, and the other weed species.

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