

Molecular Identification of an Isolate of *Peanut Mottle Virus* (PeMoV) in Iran

N. Beikzadeh^{1*}, A. Hassani-Mehraban², and D. Peters³

ABSTRACT

Peanut plants showing mottling, yellow and necrotic spots on leaves were collected from peanut fields in Golestan province. Electron microscopic studies revealed the presence of flexuous filamentous particles ca. 700 nm in length, which was suggestive of a potyvirus infection. Healthy *Nicotiana benthamiana* plants mechanically inoculated with sap from infected peanut plants showed mottling, downward leaf curling, and wrinkling of the leaves. The virus was transmitted by *Myzus persicae* in a non-persistent manner to healthy *N. benthamiana*, on which symptoms were observed two weeks later. RT-PCR using an Oligo-dT and a Nib primer set resulted in a fragment of about 1093 bp, which comprised the complete coat protein (CP) gene and 3'-non-coding region. Analysis of its CP nucleotide and amino acid sequence revealed 98-99% similarity and 95-99% identity to those of *Peanut mottle virus* (PeMoV) isolated from other countries, respectively. The molecular data confirmed serological, vector transmission, and electron microscopic findings on the incidence of PeMoV in Iran. Additionally, sequence and phylogenetic analyses of the CP revealed clustering of Iranian PeMoV isolate with Asian/Australian isolates.

Keywords: *Arachis hypogaea*, Phylogenetic analysis, *Potyviridae*.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is grown in tropical and temperate regions as oilseed crop in China, India, and the United States (Reddy, 1991). Peanut is the fourth most commonly produced oilseed in the world after soybean, rapeseed, and cottonseed (Ozudogru, 2011). In Iran, this crop is mainly grown in the provinces of Golestan, Khuzestan, and Guilan for the production of consumption nuts (Noorhosseini Niyaki and Haghdoost Manjili, 2009; Radjabi and Noorhosseini Niyaki, 2010). More than 20 viruses belonging to different virus families infect peanut naturally (Spiegel *et al.*, 2008).

Cucumber Mosaic Virus (CMV), *Peanut Stripe Virus* (PStV) and *Peanut Stunt Virus* (PSV), which are transmitted through seeds, are the most devastating viruses infecting peanut crop. These viruses are of particular economic importance in developing countries as they cause severe reductions in seed yield and quality (Spiegel *et al.*, 2008; Akin and Sudarsono, 1997; Kuhn, 1965; Xu *et al.*, 1991; Xu *et al.*, 1998). *Peanut Mottle Virus* (PeMoV), a species of the genus *Potyvirus*, occurs worldwide (Behncken, 1970; Sreenivasulu and Demski, 1988). This virus was first found to infect peanuts and soybean (*Glycine max*) in Georgia, USA (Kuhn, 1965). PeMoV is transmitted by

¹ Khorasan Razavi Agricultural Education Center, Technical and Vocational Higher Education Institute, Mashhad, Islamic Republic of Iran.

* Corresponding author; e-mail: beiczadeh@yahoo.com

² The Netherlands Food and Consumer Product Safety Authority, Section of Virology and Molecular Biology, P. O. Box: 9102, 6700 HC Wageningen, The Netherlands.

³ Department of Plant Sciences, Laboratory of Virology, Wageningen University, P. O. Box: 629, Wageningen, The Netherlands.



different aphid species like *Myzus persicae*, *Aphis craccivora*, *A. gossypi* and *Rhopalosiphum padi* (Behncken, 1970; Sreenivasulu and Demski, 1988). Its transmission by seeds is assumed to be the reason for its present worldwide distribution in peanut (Dang et al., 2010; Gillaspie et al., 2000). The virus has also been found in other important crops, such as pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) (Brunt et al., 1990).

Two viral diseases in peanut crop have been reported in Golestan province. Their symptoms resemble those earlier described for a disease assumed to be caused by PeMoV (Elahinia et al., 2008; Shahraeen and Bananej, 1995), but also a tospovirus *Groundnut Bud Necrosis Virus* (GBNV) (Golnaraghi et al., 2002). Since their first discovery, similar symptoms have been frequently observed in peanut fields in the same area. The disease on peanut was highlighted by mottling, necrosis on the leaves and necrotic areas on the shoots. Although initial detection of both viruses in Iran was mainly based on enzyme-linked immunosorbent assay (ELISA), little is known about their molecular identity. Here, we present molecular data of the viral agent of the disease using reverse transcriptase polymerase chain reaction (RT-PCR)

approach to confirm the presence of PeMoV in Iran. The sequences obtained from the coat protein and 3'-non-coding region of PeMoV are compared with other isolates described in other countries.

MATERIALS AND METHODS

Virus Source

In June 2009, peanut plants showing mottling and yellow and necrotic spots (Figure 1) were collected from five peanut fields in Golestan province. Sap from infected leaf samples was mechanically inoculated on *Petunia hybrida* (as an indicator plant for tospovirus infections) using 0.5M phosphate buffered saline (PBS), pH 7.0., containing 0.01% Na₂SO₃ (Allen and Matteoni, 1991; de Ávila et al., 1993) and *Nicotiana benthamiana* to maintain the virus isolates, and stored at -80°C for further studies.

Host Range Study

In addition to *P. hybrida* and *N. benthamiana*, crude extracts of infected peanut leaves were inoculated to a limited



Figure 1. Peanut leaves and a plant showing symptoms of PeMoV. Chlorotic areas progressing to necrotic spots on the leaves and stem are shown.

number of plant species including *Chenopodium quinoa*, *Chrysanthemum* sp., *Emilia sonchifolia*, *Cucumis sativus*, and *Capsicum annuum*.

Serological Tests

Polyclonal antisera (IgG and conjugate) raised against GBNV and PeMoV were applied to detect these viruses in the collected leaf samples and in inoculated *N. benthamiana* plants using the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977). The PeMoV serum and positive control material were kindly provided by Dr. S. Winter, Leibniz Institute DSMZ, Braunschweig, Germany. The GBNV reagents were used in a dilution of 1 $\mu\text{g mL}^{-1}$ and the PeMoV reagents in a dilution of 2 $\mu\text{g mL}^{-1}$. The absorbance values were measured at 405 nm by an ELISA-reader (FLUOstar OPTIMA (BMG LABTECH GmbH, Germany) after 60 minutes. Samples with absorbance values greater than or equal to three times the average of negative control were considered to be positive.

Aphid Transmission Test

Five-leaf-stage *N. benthamiana* plants were used as test plants. Virus-free green peach aphids adults, *Myzus persicae* Sulz., reared on healthy radish plants, were starved for 1 h and transferred onto infected *N. benthamiana* plants for an acquisition access feeding period of 2-5 minutes. Groups of 20 aphids were transferred to two healthy *N. benthamiana* plants. Aphids were removed after an inoculation access period of 1-2 hours. As negative control, virus-free aphids were placed on healthy *N. benthamiana* leaves and transferred to healthy plants (Sreenivasulu and Demski, 1988; Samad *et al.*, 1993). The plants were kept for 2 weeks in the greenhouse for symptom appearance and then analyzed using reverse-

transcription polymerase chain reaction (RT-PCR) assay for the presence of virus.

Electron Microscopy

Extracts of *N. benthamiana* plants with systemic mottling and downward curling of the leaves and healthy plants were used to prepare leaf dip preparations. They were negatively stained with 2% uranyl acetate (pH 3.8) and examined with a JEOL JME1011 electron microscope.

Total RNA Extraction and RT-PCR

To confirm the identification of the virus found in the samples with positive PeMoV reactions in DAS-ELISA, total RNA was extracted from 100 mg healthy and infected fresh *N. benthamiana* leaf tissue using Trizol reagent according to manufacturer's instruction (Invitrogen, USA). A primer set was applied to target the 3'-non-coding region (3'-NCR) sequence and coat protein gene (Figure 2-B). The first cDNA strand was synthesized from the purified RNA with Potyvirid Oligo-dT₍₁₇₎ [5'-CACGGATCCCGGG(T)17VGC-3'] complementary to the 3' terminal poly-A of potyvirid species with an additional *Bam*HI (underlined) sequence] and GBNV-R [5'-TTACAATTCCAGCGAAGGAC-3'] at nucleotide position 2160-2180 based on the entire sequence of the S RNA segment (Acc. No. U27809)] (Gibbs and Mackenzie, 1997; Satyanarayana *et al.*, 1996, Zheng *et al.*, 2010) using AMV-reverse transcriptase (Promega, USA) and incubated for 1 h at 42°C. To amplify the PeMoV coat protein and GBNV N genes, a forward primer annealing to nuclear inclusion B (NIb) gene (NIbFor: 5'-TGATGAAGTTCGTTACCAGTC-3') identical to nucleotide position 8567-8587 and GBNV-F (5'-ATGTCTAACGTCAAGCAAC-3') to nucleotide position 2971-2990 were designed based on the alignment of two NIb



sequences from PeMoV isolates (Acc. Nos. AF023848 and X73422) and the start codon of the N gene, respectively. PCR-amplification for both viruses was carried out using GoTaq polymerase (Promega) under the conditions: an initial denaturation step at 94°C for 2 minutes followed by 30 cycles at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension of 72°C for 60 seconds with a final extension step at 72°C for 5 minutes. This PeMoV set of primer was also used to detect the virus in the plants infected via viruliferous *M. persicae* in the transmission test.

Cloning, Sequencing and Phylogenetic Analysis

PCR products of the expected size were purified using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). The purified fragments were cloned in the pGEM-T Easy (Promega) vector and subsequently transformed into *Escherichia coli* DH5α electrocompetent cells. Isolation of recombinant plasmid DNA was carried out using GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, Germany). The sequences of the CP-NCR region PCR products were analyzed using BLASTn and p. The sequence data were aligned with the other PeMoV sequences using Clustal W alignment (Thompson *et al.*, 1994). The multiple sequence alignment of CP nt and aa sequences were used as input for construction of phylogenetic tree using the MEGA5 package neighbor-joining method (Tamura *et al.*, 2011).

RESULTS

Host Range and Serology

The virus infecting peanut was transmitted to *N. benthamiana*, *P. hybrida* and *E. sonchifolia*. Only *N. benthamiana* developed a systemic reaction displaying a faint mottling, a downward curling and wrinkling of the systemically infected leaves. Presence of PeMoV in these infected *N. benthamiana* plants was confirmed with PeMoV antiserum using ELISA. No positive reactions were observed in the samples against GBNV antiserum in ELISA (Table 1).

Electron Microscopy

EM examination revealed presence of flexuous rod particles only in the crude sap extracted from the infected *N. benthamiana* plants with an approximate mean length of 700 nm and a width of 12 nm (Figure 2-A). No enveloped tospovirus-like particle was observed.

Vector Transmission

The virus was transmitted by *M. persicae* from infected *N. benthamiana* plants to the healthy plants in a non-persistent manner. Infection of the plants was confirmed by the amplification of a specific PeMoV-specific fragment (~1,093 bp) by RT-PCR using specific primers (Figure 2-E).

Table 1. ELISA results of the test to detect PeMoV and GBNV in groundnut samples collected in Golestan.

Source	Virus dilution			
	PeMoV		GBNV	
	1:10	1:50	1:10	1:50
Diseased sample	1.582	0.955	0.068	0.057
Infected <i>N. benthamiana</i>	1.853	1.147	0.074	0.062
Positive control	1.488	1.036	0.064	0.065
Healthy leaf material	0.064	0.057	0.083	0.71

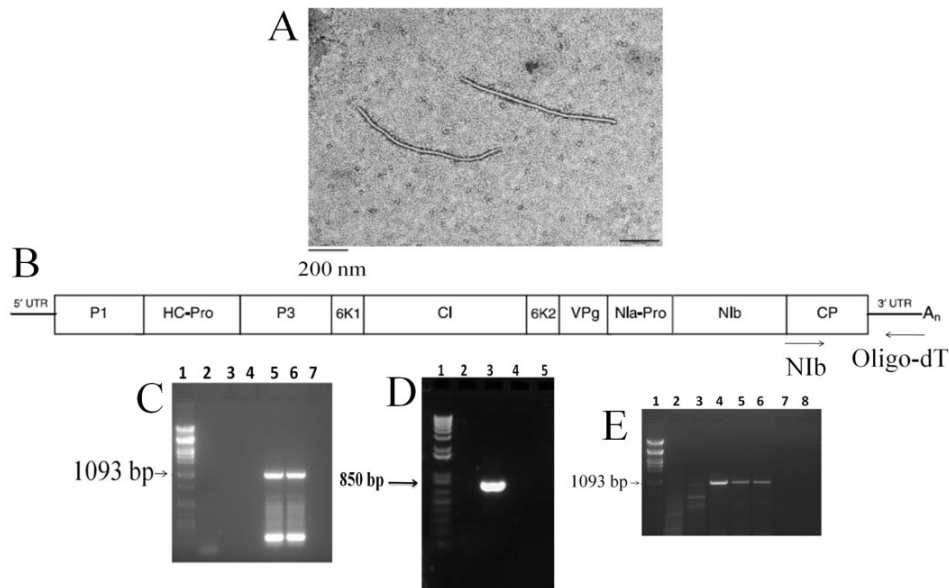


Figure 2. (A) Electron micrograph (x8000) of virus particles stained with 2% uranyl acetate (scale bar: 200 nm); (B) Schematic diagram of a potyviral genome (~10 kb). Position of primers *i.e.* potyvirid (Oligo-dT) and NlB indicated by arrows; (C) Detection of PeMoV CP in 1% agarose gel [Lane 1: Lambda DNA/PstI marker; Lane 2: Negative (healthy *N. benthamiana*) control; Lanes 3, 4 and 7: Empty, Lanes 5 and 6: Samples of two different infected plants]; (D) Detection of GBNV CP in 1% agarose gel [Lane 1: 1 Kb Plus DNA Ladder; Lane 2: Negative (healthy *N. benthamiana*) control; Lane 3: GBNV positive control, Lanes 4 and 5: *N. benthamiana* plants infected with PeMoV], and (E) RT-PCR detection of PeMoV in *N. benthamiana* plants infected via *M. persicae* transmission (Lane 1: Lambda DNA/PstI marker; Lanes 2, 7 and 8: Empty; Lane 3: Negative control; Lane 4: Positive control, Lanes 5 and 6: Samples of two different infected plants).

Sequence and Phylogenetic Analysis

RT-PCR using PeMoV NlB and Oligo-dT primers resulted in amplification of a PeMoV-specific fragment (~1,093 bp) containing a part of NlB protein gene, the complete coat protein (CP) gene and the 3'-NCR (284 nts) followed by a poly-A tail. No products were obtained in the healthy control (Figure 2-C). Furthermore, GBNV was not detected in the original samples and of those *N. benthamiana* plants inoculated with these samples and with PeMoV (Figure 2-D). The amplicon (~1,093 bp) was cloned, sequenced and submitted to the GenBank (Acc. no. JX441319). Blast analysis of the CP and 3'-NCR sequences confirmed that the diseased plants were indeed infected by PeMoV. The nucleotide sequences of the

entire CP and part of 3'end of NlB gene of the isolate consisted of 853 nts encoding a protein of 283 deduced amino acid (aa) residues with MW 32.2 kDa. The nucleotide sequences of the CP gene, the 3'-NCR, and the deduced aa sequences of CP (278 aa residues) of the new isolate were compared with those of previously reported PeMoV isolates. Based on this analysis, the PeMoV isolate (designated PeMoV-IR) shared 99% nucleotide sequence identity with the CP gene of the isolates M (Acc. no. AF023848), PV4 (Acc. no. L32959) and T (Acc. no. L32960) from the USA, 98% with an isolate from Israel (Acc. no. DQ868539), Australia (Acc. no. X73422), China (DQ6; Acc. no. GQ180068), India (Gn-Hyd-1; Acc. no. JX088125) and three isolates from USA (AR, 3b8 and M; Acc. nos. L32956, L32957 and L32958, respectively) and 97% with the Chinese isolate (DQ5; Acc. no. GQ180067).



The CP amino acid sequence shared an identity of 99% with M (Acc. no. AF023848), PV4, DQ5, DQ6, Gn-Hyd-1, the Israeli and Australian, 97% with T and M, 96% with AR, and 95% with 3b8 isolates.

The 3'NCR sequences of PeMoV-IR and the isolates M, PV4, AR, T, 3b8 and the Australian shared more than 96% nt sequence identity (Table 2). The length of these 3'-NCRs varied from 284 (PeMoV-IR)-290 (M isolate; Acc. no. AF023848) nts due to some deletions.

To determine the phylogenetic relationship of the PeMoV-IR isolate with the eleven other known isolates, their coat protein aa and nt sequences were used to construct the corresponding phylogenetic trees. The obtained phylogenetic tree based on aa sequences clearly showed that the PeMoV isolates were clustered in two distinct groups (Figure 3). One group contained isolates that were isolated from peanut and *Pisum sativum*, while the second group contained only isolates from *P. sativum*. High bootstrap values confirmed the grouping of the PeMoV-IR, Israeli, Australian, DQ5, DQ6, Gn-Hyd-1 and both M isolates on the same branch.

DISCUSSION

In this study, we identified PeMoV as the causal agent in peanut plants showing virus-like symptoms. Evidence was obtained for a potyvirus etiology by aphid transmission, electron microscopy, and host range studies. We confirmed its identity as being PeMoV by serological studies (ELISA), RT-PCR, and phylogenetic analyses. With these results, we confirm an earlier conclusion made by Elahinia *et al.* (2008) and Shahraeen and Bananej (1995) that PeMoV occurs in Iran.

The symptoms on the collected peanut plants also resembled those caused by GBNV (Reddy *et al.*, 1995). The absence of a positive reaction in ELISA using GBNV antiserum and in RT-PCR analyses indicated

Table 2. Identity (%) of the 3'-UTR nucleotide sequence among the studied PeMoV isolates.

PeMoV isolates	JX441319	L32959	AF023848	DQ868539	X73422	GQ180068	GQ180067	L32958	L32960	L32956	L32957	JX088125
JX441319(Iran)	100	97.89	97.54	*	94.72	*	*	96.48	97.54	97.89	96.83	*
L32959(PV4)		100	99.65	*	97.55	*	*	98.62	99.65	100	98.96	*
AF023848(M)			100	*	97.2	*	*	98.96	99.31	99.65	98.61	*
DQ868539(Israel)				100	*	*	*	*	*	*	*	*
X73422(Australia)					100	*	*	96.5	97.2	97.55	96.15	*
GQ180068(DQ6)						100	*	*	*	*	*	*
GQ180067(DQ5)							100	*	*	*	*	*
L32958(M)								100	98.27	98.62	97.57	*
L32960(T)									100	99.65	98.96	*
L32956(AR)										100	98.96	*
L32957(3b8)											100	*
JX088125(Gn-Hyd-1)												100

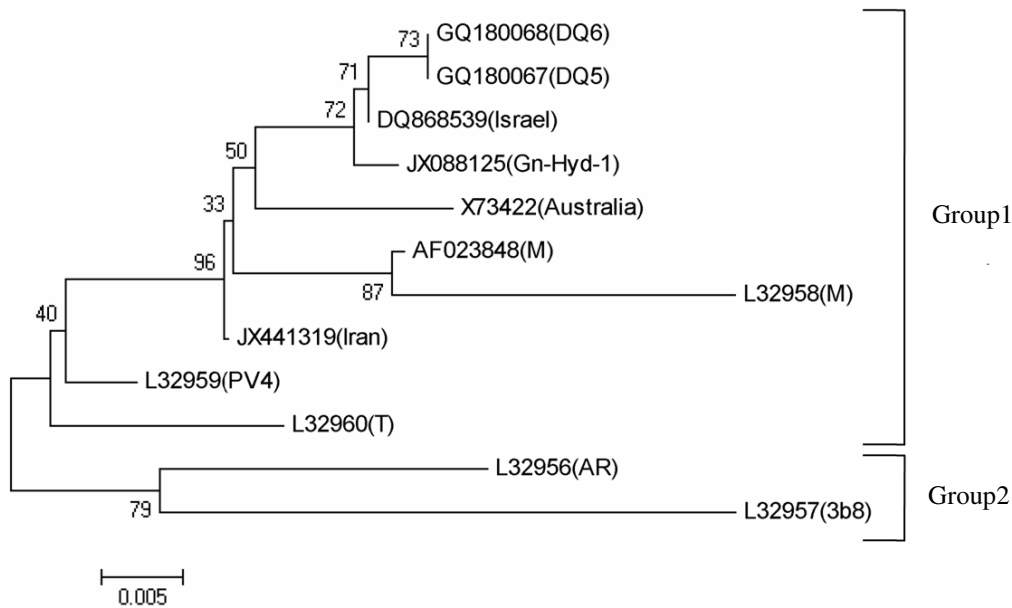


Figure 3. Phylogenetic tree was constructed by the neighbor-joining method from multiple sequence alignments of 12 PeMoV isolates using aa sequences of coat protein. Bootstrap values (percentage of 1,000 replications) are shown for the relevant nodes.

that the samples were not mixed-infected with GBNV. No necrotic lesion symptoms on inoculated *P. hybrida* appeared as expected upon GBNV infection (Reddy *et al.*, 1995). This tospovirus exists in South and Southeast Asia, and has been reported in the Golestan province in Iran (Golnaraghi *et al.*, 2002). In our study, we did not find any evidence for the presence of GBNV in our samples (Table 1 and Figure 2-D).

The PeMoV-IR symptoms showed some similarities to the naturally occurring N isolate in the USA i.e. causing initially chlorotic spots on young developing leaflets which became necrotic two to three days later (Paguio and Kuhn, 1973). Slight differences in symptom expression between PeMoV-IR and the isolates found in other countries can be noticed (Sun and Hebert, 1972; Paguio and Kuhn, 1973; Samad *et al.*, 1993; Elahinia *et al.*, 2008; Shahræen and Bananej, 1995; de Breuil *et al.*, 2008; Ahmed and Idris, 1981; Spiegel *et al.*, 2008). This slight difference may be due to the infecting isolate, the cultivar used, the growing condition, and the judgment of the observer, etc. Mixed-infections of *Cucumber*

mosaic virus and PeMoV-M strain can cause synergistic reactions in California on blackeye cowpeas resulting in necrosis and stunting (Demski *et al.*, 1983). High temperatures might also have an effect on symptom expression and the development of necrosis symptoms. Peanut plants kept at 16°C for one month did not develop necrotic symptoms, but when transferred to 26-32°C, the new growth of these plants responded with necrotic symptoms (Paguio and Kuhn, 1973).

Potyvirus Oligo-dT₍₁₇₎ and NibFor primers were used to amplify the CP and the 3'-NCR commonly used as markers to differentiate potyviruses (Bousalem and Loubet, 2007). Species in *Potyviridae* are distinguished by a CP aa sequence identity less than about 80% and a nt sequence identity less than 76% either in the CP or over the whole genome (Adams *et al.*, 2012). According to the criteria used, PeMoV-IR should be considered as an isolate of PeMoV.

Phylogenetic analysis of the amino acid sequences of the coat protein PeMoV isolates can be splitted into two groups, which are partially correlated with the



geographical origin of the isolates and probably in the natural host infected. Based on these results (Figure 3), the Iranian, Australian, Israeli, DQ5, DQ6, Gn-Hyd-1 and both M PeMoV isolates may have a common ancestor, although to support this conclusion more isolates of PeMoV in Iran, USA and other Asian countries need to be sequenced.

The NCR sequences are used as basis for identifying and classifying potyviruses (Frenkel et al., 1989; Abdullah et al., 2009, Adams et al, 2012). These sequences of different potyvirus species display a high degree of sequence variability (39-53%), whereas the homology between strains of the

same virus species generally ranges from 83-99% (Frenkel et al., 1989; Van der Vlugt et al., 1993). Pairwise sequence alignments of 3'-NCR nucleotide sequences (Table 2) showed 94.7-97.9% homology between the Iranian and the other PeMoV isolates.

The highly conserved Asa-Ala-Gly (DAG) motif, found in many aphid-transmissible potyviruses (Atreya et al., 1990), is replaced in the PeMoV isolates listed above with a DAA motif, including our PeMoV-IR isolate (Figure 4), which seems unique for PeMoV (Spiegel et al., 2008). The three consensus motifs, MVWCIENGTSP, AFDF and QMKAAAL have been found in the CP of most potyviruses (Dujovny et al., 2000). MVWCIENGTSP was

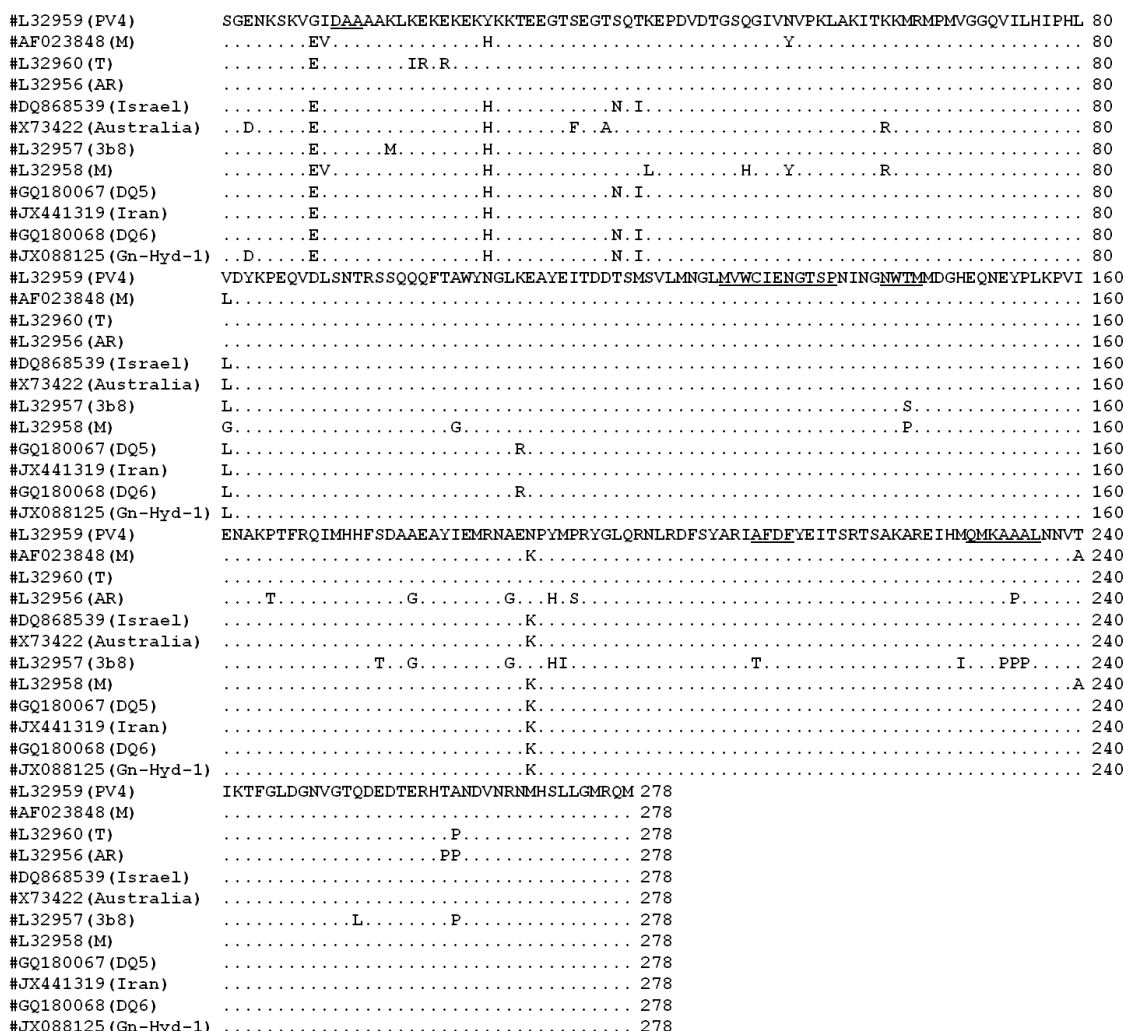


Figure 4. Multiple sequence alignment of the twelve PeMoV isolates based on coat protein amino acid sequences.

found in all PeMoV isolates discussed in this study. However, AFDF changed to TFDF and QMKAAAL to QMKPPPL in the strain 3b8 and to QMKAPAL in the strain AR (Figure 4). PeMoV-IR CP, like other PeMoV isolates, has two NT (asparagine-threonine) rich potential N-glycosylation motif sequences (NGTS and NWTM) in the central region (Figure 4). Similar motifs were also found in CP of three potyviruses, viz. *Dasheen mosaic virus* (Pappu *et al.*, 1994), *Iris severe mosaic virus* (Park *et al.*, 2000) and *Ornithogalum mosaic virus* (Yoon and Ryu, 2002) and the potyvirus *Potato virus X* (Tozzini *et al.*, 1994). These motives are commonly found in glycoproteins of some animal viruses and are considered to be essential for host membrane recognition and plant-virus interaction (Zaret and Sherman, 1982; Yoon and Ryu, 2002).

Detection of PeMoV (present report) and also of GBNV (Golnaraghi *et al.*, 2002) in the Golestan province indicates that both viruses can form a threat for the groundnut industry in this province. This may be also true for the groundnut industry of Khuzestan and Guilan provinces, in which, apparently, these viruses have not been detected to our knowledge. Control of the spread of these viruses transmitted by aphids and thrips will be difficult. However, we like to advocate a risk assessment method to facilitate an integrated pest management method as developed by Brown *et al.* (2005) in Georgia, USA, resulting in an evident reduction in the spread of *Tomato Spotted Wilt Virus*. These scientists assessed the risk of incidence using different factors like groundnut cultivar, planting date, plant density, use of insecticides, infection history of the farms, row pattern, tillage, and herbicide application. The evaluated risks were given indices. Depending on assessment, the risk categories including low, moderate, and high were distinguished. Application the risk resulted in considerable reduction in the incidence. To this end, studies might be initiated to detect sources of PeMoV and GBNV in the field, the susceptibility of the peanut cultivars used in Iran, and farming practices like planting date, tillage, and plant spacing.

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شناسایی مولکولی جدایه ای از ویروس ابلقی بادام زمینی (*Peanut mottle virus*; PeMoV)

در ایران

ن. بیک زاده، ا. حسنی مهربان، د. پترز

چکیده

بوته های بادام زمینی با علائم ابلقی، زردی و لکه های بافت مرده روی برگ ها، از مزارع بادام زمینی در استان گلستان جمع آوری گردید. مطالعات الکترون میکروسکوپی، وجود ذرات رشته ای خمش پذیر با طول تقریباً ۷۰۰ نانومتر را نشان داد و بر این اساس آلودگی این نمونه ها به یک پوتی ویروس



پیشنهاد شد. بر روی بوته های سالم *Nicotiana benthamiana* که به طریق مکانیکی با استفاده از عصاره تهیه شده از بوته های آلوده بادام زمینی مایه زنی شده بودند، علائم ابلقی، پیچیدگی برگ ها به سمت پایین و موجدار حاشیه برگ ها ظاهر گردید. این ویروس با استفاده از شته سبز هلو (*Myzus persicae*) با روش غیرپایا به بوته های سالم *N. benthamiana* منتقل و دو هفته پس از آن، علائم آلودگی ظاهر شد. با استفاده از آزمون RT-PCR و آغازگرهای Oligo-dT و Nib، یک قطعه در حدود ۱۰۹۳ جفت باز که شامل ناحیه غیر کد شونده در انتهای ۳' و ژن پروتئین پوششی به طور کامل بود، تکثیر گردید. تجزیه و تحلیل توالی نوکلئوتیدی و آمینواسیدی این پروتئین پوششی نشان داد که با توالی نوکلئوتیدی و آمینواسیدی پروتئین پوششی جدایه های ویروس ابلقی بادام زمینی (*Peanut mottle virus; PeMoV*) از سایر کشورها به ترتیب ۹۹-۹۸ درصد و ۹۹-۹۵ درصد شباهت دارد. این داده های مولکولی، نتایج به دست آمده از آزمون سرم شناسی، انتقال با ناقل و الکترون میکروسکوپی در مورد وقوع ویروس ابلقی بادام زمینی در ایران را تایید می کند. علاوه بر این، آنالیز توالی و فیلوژنتیکی پروتئین پوششی نشان داد که این جدایه ایرانی با جدایه های آسیایی / استرالیایی، گروه بندی می شود.