Nucleotide sequence analyses of coat protein gene of peanut stunt virus isolates from alfalfa and different hosts show a new tentative subgroup from Iran

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Abstract Alfalfa cultivars grown in 14 provinces in Iran were surveyed for the relative incidence of peanut stunt virus (PSV) during 2013–2016. PSV were detected in 41.89% of symptomatic alfalfa samples and a few alternate hosts by plate-trapped antigen ELISA. Among other hosts tested only *Chenopodium album*, *Robinia pseudoacacia* and *Arachis hypogaea* were found naturally infected with PSV. Twenty five isolates of PSV were chosen for biological and molecular characterizations based on their geographical distributions. There was not any differences in experimental host range of these isolates; however, variation in systemic symptoms observed on *Nicotiana glutinosa*. Total RNA from 25 of viral isolates were subjected to reverse transcription polymerase chain reaction analysis using primers directed against coat protein (CP) gene. The CP genes of 25 Iranian PSV isolates were either 651 or 666 nucleotides long. The nucleotide and amino acid identities for CP gene among Iranian PSV isolates were 79.3–99.7 and 72–100%, respectively. They also shared between 67.4 and 82.4% pairwise nucleotide identity with other PSV isolates reported elsewhere in the world. Phylogenetic analyses of CP gene sequences

showed formation of a new subgroup comprising only the Iranian isolates. Natural infection of a few alternate hosts with PSV is reported for the first time from Iran.

Keywords Coat protein gene · Phylogeny · Peanut stunt virus

Introduction

Peanut stunt virus (PSV) is a member of the genus *Cucumovirus* within the family *Bromoviridae* [28]. The virus was first reported in USA in 1964 [22, 36]. PSV is transmitted by aphids in non-persistent manner and has a worldwide distribution [28]. PSV causes economically important diseases mostly on legumes such as alfalfa (*Medicago sativa* L.) [7], red clover (*Trifolium pratense* L.) [5], Kura clover (*Trifolium ambiguum* L.), white clover (*Trifolium repens* L.) [1], peanut (*Arachis hypogaea* L.) [36], pea (*Pisum sativum* L.) [17], bean (*Phaseolus vulgaris* L.) [9], soybean (*Glycine max* L. Merr.) [20], yellow lupine (*Lupinus arboreus* Sims) [10] and black locust tree (*Robinia pseudoacacia* L.) [4, 17]. Its host range also includes tomatoes (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana* spp.) [11], peppers (*Capsicum annuum* L.), cucurbits (*Cucumis sativus* L. and *Cucurbita pepo* L.) [2].

PSV has a genome composed of three positive-sense RNA molecules. RNA1 and RNA2 are monocistronic and RNA3 is bicistronic [33]. RNAs1 and 2 encode the viral replicase complex consisting of proteins 1a and 2a, synthesized from RNA1 and RNA2, respectively. Proteins 1a has two motifs, one is helicase motif residing on C-terminus whereas the second motif that is methyltransferase located at its N-terminus [32]. Protein 2a has one RNA-

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dependent RNA polymerase motif (RdRp) [18]. RNA3 encodes 3a protein, the viral movement protein, and the coat protein (CP) that is expressed from a subgenomic RNA (RNA4) and is packaged together with RNA3 in a single viral particle [25].

Over the last decade, based on host range, particle stability, immunological relationships and competition hybridization analyses PSV strains were placed into Eastern (E-type) and Western (W-type) type strains [8, 23]. These two groupings were further reaffirmed based on support for satellite RNA replication and sequence homologies detected by Northern blot analysis [26]. Subsequently, more diversity in PSV isolates belonging to the E-type was shown in comparison to those isolates belonging to W-type [37]. Nucleotide sequence analysis of PSV RNA 3 also showed that PSV strains are classified in E-type and subgroup II W-type [13]. Later, a third subgroup was proposed based on the complete nucleotide sequence of a Chinese PSV strain (PSV-Mi) representing its type isolate [37, 38]. Subsequently, based on nucleotide sequence analysis of the PSV isolates from *Robinia pseudoacacia*, a fourth PSV subgroup was proposed [16, 17]. It should be noted that evidence has been presented that the phylogenetic relationships among PSV strains are even more complex than what has already been reported [14, 21, 31].

Alfalfa is a crop growing for the purpose of producing forage in various regions of Iran for a long time. In fact, most authorities generally agree that alfalfa probably originated in Persia (now mainly Iran) [6]. Currently alfalfa is widely grown in different parts of Iran with the exception of three western provinces [30]. PSV was reported for the first time in Iran on the basis of biological, serological and physico-biochemical properties by Bananej et al. [3]. Following further characterization of this isolate based on Northern blot hybridization by Hajimorad et al. [12], it was placed in subgroup II.

The objectives of this research were (1) to find out the distribution of PSV in alfalfa growing regions of Iran; (2) to characterize naturally occurring PSV isolates from Iran; and (3) to investigate phylogenetic relationship of Iranian PSV isolates based on CP gene sequences in comparison with those reported elsewhere in the world.

Materials and methods

Collection and maintenance of virus isolates

Alfalfa producing areas located in northwest (West Azerbaijan), north (Gilan, Mazandaran, Golestan), northeast (Razavi Khorasan), center (Yazd and Isfahan), west (Lorestan), southwest (Khuzestan, Kohgiluyeh and Boyer-

Ahmad), south (Hormozgan and Shiraz) and southeast (Kerman and Sistan and Baluchestan) (see details in Supplementary Fig. 1) were surveyed for the presence of PSV. A total of 1270 samples comprised of alfalfa ($n = 986$) and other plant species ($n = 284$) were collected from 64 locations between January 2013 and July 2016 (see details in Supplementary Table 1). In general, samples were collected from plants exhibiting one or a combination of viral-like symptoms including mosaic, mild to severe mottling, vein banding and leaf malformation. All samples were screened for PSV by plate-trapped antigen (PTA) ELISA [24]. PSV antiserum was prepared in our laboratory in Department of Plant Protection, Shahid Bahonar University of Kerman (data not shown). Positive and negative controls from previous research [19], were included in all screening tests. In all ELISA assays a sample was considered virus-positive only if its optical density (OD) at 405 nm exceeded the mean value plus three standard deviations of the OD of the healthy controls.

A subset of 25 PSV isolates were selected according to hosts and agroecological considerations to highlight the maximum potential variability and maintained on *Nicotiana glutinosa* L., in a temperature-regulated insect-proof greenhouse for subsequent studies. These isolates were assigned a name according to the abbreviated names of the province, region, and the host plant from which isolated (see details in Supplementary Table 2).

Host range study

Leaf tissues from *N. glutinosa* systemically infected with PSV isolates was ground in 1% (w/v) solution of K_2HPO_4 at pH 7.5 containing 0.01% Na_2SO_3 , 2% polyvinylpyrrolidone (PVP) and 0.05% ethylene diamine tetraacetic acid (EDTA). The extracts were then mechanically inoculated onto the following Carboruandum-dusted plant species: *N. clevelei* Gray., *N. tabacum* L.cv. Samsun.NN, *N. tabacum* L. cv. White burley, *N. glutinosa* L., *N. turkish* L., *N. rustica* L., *Datura stramonium* L., *Glycine max* L., *Phaseolus vulgaris* L.cv. Red Kidney, *Chenopodium quinoa* Willd, and *Gompherena globosa* L. Inoculated plants were maintained in greenhouse at room temperature and examined regularly for symptom expression and assayed for the presence of PSV by PTA-ELISA.

Reverse transcription (RT)-polymerase chain reaction (PCR) and cloning

Total RNA was extracted from the young leaves of the PSV infected *N. glutinosa* plants using the High Pure Viral Nucleic Acid kit (Roche Biochemical, Germany). The first-strand cDNA synthesis was performed using Moloney murine leukemia virus (M-MuLV) reverse transcriptase

(Thermo Fisher Scientific, USA) as described by Sharifi et al. [35]. A set of specific primers PSV-F8/PSV-R8 [19] were used to amplify CP gene of 25 Iranian PSV isolates in the presence of *Taq* DNA polymerase (Fermentas, Lithuania).

The PCR program consisted of an initial denaturation for 3 min at 94 °C, followed by 45 s at 94 °C, 60 s at 52, 72 °C for 90 s (35 cycles) and a final extension for 10 min at 72 °C. The amplified product was analyzed by gel electrophoresis and then PCR amplicons in the most cases for sequencing were purified using High Pure PCR product purification kit (Roche, Germany) according to the manufacturer's instructions. In some cases, PCR products were ligated into the pTZ57R/T plasmid using InsT/A clone PCR Product Cloning Kit (Thermo Fisher Scientific) according to the manufacturer's recommendation. Recombinant plasmids were extracted from transformed *Escherichia coli* strain DH5a and digested by *Eco*RI and *Pst*II restriction enzymes (Thermo Fisher Scientific) to screen for the presence of insert.

PCR products or two independent recombinant plasmids for each insert were sequenced on both strands at Bioneer Company (South Korea) using automatic sequencer 37370XI with specific primers PSV-F8/PSV-R8 [19] or M13 forward and reverse primers, respectively.

The deduced amino acid (aa) sequences was generated with DNAMAN (Lynnon, Biosoft, Quebec, Canada) protein analysis program. Maximum likelihood phylogenetic trees of CP from PSV isolates were constructed using MEGA6 tool with 1000 replicates. Nucleotide (nt) and amino acid (aa) pairwise identities were calculated using SDT_v 1.0 and DNAMAN programs. Genetic distances were estimated from sequence data by means of the Kimura two-parameter method [15] using MEGA version 6 program. To estimates d_N and d_S values, DnaSP version 4.0 was used [34].

Results

Surveys of alfalfa fields for PSV

Of the 1270 samples collected from alfalfa fields and alternate hosts, which assayed by ELISA, a total of 532 (41.89%) were infected with PSV. PSV infection was confirmed in naturally infected *C. album*, *Robinia pseudoacacia* and *Arachis hypogaea* collected from fields (Fig. 1).

Host range of PSV isolates

Overall, the symptom and host range on selected experimental plants for all isolates were similar (Table 1). PSV

isolates induced systemic mosaic and general chlorosis on *N. tabacum* cv. rustica L., *N. tabacum* cv. Samsun, *N. clevelandii* Gray and *D. stramonium* L. They also infected *N. tabacum* L. cv. White burley systemically causing mild mosaic and showed only systemic mosaic on *N. tabacum* L. cv. Turkish. In contrast to solanaceous plants, Iranian PSV isolates induced chlorotic local lesions and systemic symptoms of mosaic and general chlorosis in *C. quinoa*. Inoculated *P. vulgaris* cv. Red kidney and *G. max* L. produced necrotic local lesions and mild mosaic. None of the isolates showed any signs of infection on *Gomphrena globosa* L. It is worth to note that the inoculated PSV isolates onto *N. glutinosa* L., induced a range of symptoms that included systemic mosaic, malformation and leaf narrowing.

Variability of CP-encoding regions of 25 Iranian PSV isolates

The complete nucleotide sequence of the CP gene for 25 Iranian PSV isolates was determined. Sequencing results showed that CP gene of ten isolates (Kz.Be.A1, Kz.Be.A2, Ke.Ke.A1, Ke.Ke.A2, Ke.Ke.Ro, Fa.Fs.A1, Fa.Fs.A2, Gi.As.Ar, Gi.As.Ch, Gi.Ra.Ch) comprised of 651 nts encoding a putative protein of 216 aa long comparable to those reported for the CP gene of some of the previously characterized PSV isolates [16, 28]. The CP gene of the remaining 15 Iranian PSV isolates contained 666 nts (with 15 additional nts) similar to CP of those previously reported Iranian PSV isolates currently available in GenBank (KF800738–KF800742) [30].

The nucleotide sequence of the CP gene of 25 PSV isolates were deposited into GenBank and assigned accession numbers KY094941–KY094965 (see details in Supplementary Table 2). The geographical origin and natural hosts for the virus isolates used in this study have been provided in Supplementary Table 2. Pairwise nucleotide sequence identity of CP gene among 25 Iranian PSV isolates ranged from 79.3 to 99.7%. The most distant isolate was Gi.Ra.Ch which was recovered from *C. album*. Amino acid identity of Iranian isolates was 72–100%, with the highest divergence between Gi.Ra.Ch (KY094963) and Kz.Be.A6 (KY094945) isolates. Furthermore, the identity levels for nucleotides and amino acids between Iranian and non-Iranian isolates ranged between 67.4–82.4% and 55.8–81.8%, respectively.

Phylogenetic analysis of the CP

Phylogenetic analysis using the CP sequences showed that 25 Iranian PSV isolates are classified into two major subgroups. Interestingly, five Iranian PSV isolates (Kh.Jo.A1, Kh.Jo.A2, Is.Jo.A, Kz.Be.A6 and Kz.Be.A3), which were

Fig. 1 a–f Symptoms of natural infections of alfalfa, and other alternate hosts with peanut stunt virus. **a** Yellowing symptoms on *Arachis hypogaea* leaf collected from Gilan, Iran; **b** symptomless *Chenopodium album* collected from Gilan, Iran; **c** mosaic, yellowing and vein banding symptoms on *Medicago sativa* collected from

Razavi Khorasan, Iran; **d** mosaic, yellowing and vein banding symptoms on *Medicago sativa* collected from Yazd, Iran; **e** Yellowing, and **f** vein banding and blistering on *Robinia pseudoacacia* tree and a dissociated leaf, respectively, collected from Kerman, Iran

recovered in the current study from *Medicago sativa* L., along with four other Iranian isolates (accession numbers KM823526 to KM823529), previously submitted to GenBank, clustered into a new tentative subgroup V. They shared identities of 87.5–99.8% and 88.2–97.3% at nt and aa levels, respectively. The other remaining 20 isolates, obtained from *M. sativa* L., *C. album*, *R. pseudoacacia* and *A. hypogaea*, clustered together with PSV-W (accession number U31366.1) and may be regarded as isolates belonging to the subgroup II (Fig. 2). They shared similarities of 83.2–99.8% and 74.3–99.5% at nucleotide and amino acid levels, respectively. The nucleotide and amino acid similarities between these two subgroups were 77.2–94.3% and

79.9–95.5%, respectively. The nucleotide sequence identity with other subgroups were as follows: subgroups I (nt: 68.2–78.6%, aa: 81–99.5%), subgroups III (nt: 67.1–76.7, aa: 98.6%), subgroups IV (nt: 70–81.8%, aa: 96.8–99.5%) (see details in Supplementary Figs. 2 and 3).

Discussion

The results of our survey conducted during 2013–2016 showed that alfalfa serves as one of the major natural hosts for PSV in Iran. Previous study showed that *Alfalfa mosaic virus* (AMV) was the main prevalent virus in the alfalfa

Table 1 Symptoms induced by Iranian isolates of peanut stunt virus on selected experimental plants following mechanical inoculation

Families, species and cultivars	Symptoms ^a
Solanaceae	
<i>Nicotiana glutinosa</i> ^b L.	SM, M, LN, GC
<i>N.tabacum</i> L. cv. White burley	MM, GC
<i>N.tabacum</i> cv. rustica L.	SM, GC
<i>N.tabacum</i> cv. Samsun NN	SM, GC
<i>N.clevelandii</i> Gray	SM, GC
<i>N.tabacum</i> L.cv. Turkish	SM
<i>Datura stramonium</i> L.	SM, GC
Chenopodiaceae	
<i>Chenopodium quinoa</i> wild	CLL, GC
Leguminosae	
<i>Phaseolus vulgaris</i> cv. Red kidney	NLL
<i>Glycine max</i> L.	NLL, MM
Amarantaceae	
<i>Gomphrena globose</i> L.	–

SM sever mosaic, M malformation, LN leaf narrowing, MM mild mosaic, GC general chlorosis, CLL chlorotic local lesions, NLL necrotic local lesions, – no symptoms and no virus recovered

^a Symptoms exhibited on Ke.Ke.A1 isolate and all other isolates showed similar symptoms

^b Variation in systemic symptoms was observed

felids in Iran [19]. In the current extensive survey, the wide distribution of PSV was confirmed in Iran as well. Its incidence varied from high in Isfahan (53.3%) and West Azerbaijan (53.5%) to moderate in Lorestan (22%) and Kohgiluyeh and Boyer-Ahmad (27.9%) and to low in Mazandaran (10.5%) provinces.

In our study, natural infection of *C. album*, a weed species, collected from alfalfa fields, was confirmed (see details in Supplementary Table 2). Therefore, this weed species may serve as a potential alternate source for spread of PSV to alfalfa. Furthermore, for the first time it was shown that *A. hypogaea* and *R. pseudoacacia* are also infected naturally with PSV; hence, they could also serve as alternate hosts for PSV. PSV is a diverse virus species and until now extensive studies have been conducted to classify PSV isolates into four subgroups [8, 14, 16, 17, 37, 38]. In this study, a phylogenetic tree was created using maximum likelihood (MEGA 6) method for 651–666 nucleotide sequences of the CP gene of 25 Iranian isolates and analyzed along with most CP sequences of PSV isolates available in GenBank ($n = 32$). In agreement with the published data [14, 16, 17, 38, 39], four PSV subgroups (I, II, III and IV) were observed (Fig. 2). According to previously established groupings, the 20 Iranian isolates from this study were all clustered in subgroup II. Five previously characterized Iranian PSV isolates [30] also were grouped in this subgroup. Based on

pairwise identities (>96% identity) coupled with maximum likelihood phylogenetic, five isolates from the current study together with four previously characterized Iranian isolates (Acc. Nos. KM823526–KM823529) were clustered in a new subgroup, which we tentatively named subgroup V. Bananej et al. [3] previously reported an Iranian PSV isolate (PSV-I). Sequence analysis of parts of RNA1 and 2 by Hajimorad et al. [12] showed that PSV-I is more closer to the W strain than to the E strain; nevertheless PSV-I could not be placed into subgroup II and consequently was named ‘atypical old world’ strain of the virus. According to our result in the current study, PSV-I isolate may be a candidate for placement into tentative subgroup V as well. However, due to lack of information about RNA3 of PSV-I more studies is needed to elucidate its relationship with other isolates in the tentative subgroup V.

The CP gene of Iranian PSV isolates from the current study and those of four previously reported Iranian isolates by Pourrahim and Farzadfar [30] (Acc. Nos. KM823526–KM823529), all had additional 15 nucleotides. The full-length CP gene of these isolates comprised of 666 nucleotides instead of 651. Nucleotide sequence of the full-length CP gene of Iranian isolates aligned well with those of other PSV strains available in GenBank. The alignment revealed that all the Iranian PSV isolates characterized in the current study and previously reported Iranian isolates [30] (KF800738–KF800742) plus American PSV-W (Acc. No. U31366.1) which clustered in subgroup II missed 3 nucleotides at proximity of the 5′ end of the CP gene. Analysis also showed that the nine Iranian isolates which clustered in tentative subgroup V contain additional 15 nucleotides at the 5′ end of the CP gene.

Hu et al. [13] proposed that PSV isolates within the same subgroup should have more than 90% sequence identity, while the nucleotide sequence identity lower than 80% is demarcation threshold to create a separate subgroup. The results in the current study indicate that minimum and maximum percentage value for nucleotide sequence identity between PSV strains clustered in subgroups I, II, III, IV and the tentative subgroup V were estimated 85.6–99.5, 83.2–99.8, 98, 96.5–99.5 and 87.5–99.8%, respectively. Therefore these observations are not in agreement with the proposal made by Hu et al. [13]. Prior to this study, reported PSV isolates from *A. hypogaea* and *R. pseudoacacia* were clustered in subgroups III [39] and IV [17], respectively. Interestingly, in the current study two Iranian isolates from *A. hypogaea* and *R. pseudoacacia* were both grouped in subgroups II. Since the majority of Iranian isolates classified in subgroups II, it is likely that these isolates may have been transmitted from alfalfa to *A. hypogaea* and *R. pseudoacacia* by aphids. In order to analyze whether geographical populations could constitute a selection factor, nucleotide sequence diversity levels

Fig. 2 Phylogenetic tree created by neighbor joining Maximum likelihood using nucleotide sequence of coat protein gene of peanut stunt virus isolates reported from around the world. *Cucumber mosaic virus* (NC_001440) used as outgroup in this Phylogenetic tree

Table 2 Average nucleotide substitution in coat protein gene and genetic diversity of peanut stunt virus isolates grouped geographically or phylogenetically

Geographical populations/Phylogenetic groups	Number of sequence	DS ^a	DN ^b	DN/dS	Genetic diversity
World	53	0.33828	0.13061	0.386	0.179
Iran	30	0.22861	0.03632	0.159	0.081
Asia	5	0.56224	0.10741	0.191	0.199
America	4	0.35075	0.09307	0.123	0.144
Europe	14	0.27546	0.04731	0.265	0.099
Subgroup I	11	0.29298	0.05954	0.203	0.115
Subgroup II	22	0.12703	0.02548	0.201	0.05
Subgroup III	2	0.03084	0.00620	0.201	0.012
Subgroup IV	11	0.04854	0.01630	0.336	0.025
Tentative subgroup V	9	0.22520	0.03541	0.157	0.079

^a Synonymous substitution^b Non-synonymous substitution

within different geographical populations and subgroups of isolates as a distinct population were calculated using the MEGA version 6 program on alignments generated from the CP gene. The intra-group diversity within Iranian isolates was 0.081, which was lower than Asian (0.199), American (0.144) and European (0.099) (Table 2). Based on four subgroups and tentative subgroup V of PSV isolates in phylogenetic tree the intra-group diversity for the three groups were lower ≥ 0.05 (in groups II, III and IV) and higher ≤ 0.079 (in group I and tentative subgroup V) (Table 2).

Negative selection due to functional constraints on virus-encoded proteins can limit the extent of genetic variation in virus populations. Therefore, we estimated the degree and sense of selection by calculating the ratio of nucleotide diversity at nonsynonymous (d_N) to synonymous (d_S) as described by Palimo and Bianchi [29], of CP coding regions among isolates from different geographical origins or different groups. High d_S in the Asian isolates (0.56224) represented the old population of this virus in the East and the role of the negative selection in the evolution of this virus. The d_S value in the population of Asia are about 0.56224 and in Europe is 0.27546 that is gradually reduced to the West. Therefore, genetic diversity of PSV strains from East to West has been decreased. As it has been shown in Table 2, replacement of synonymous substitution, (d_S) with replacement nonsynonymous substitution (d_N) is much greater. So, nucleotide changes in the population or group that is tolerated is indicative of tolerance and high plasticity of this virus.

In this study, we surveyed various alfalfa fields in Iran for PSV infection. We noted that throughout our samplings there are multiple instances of co-infections of different cultivars of alfalfa with both PSV and AMV (data not shown). We

noticed that CP gene from the Iranian isolates falls into two different sequence lengths (651 or 666 nucleotides); however, we were unable to find any correlation between the sequence length variation and phylogenetic relationships. Hence, there is a clear need for further studies to identify the role (s) of variation in the length of the coding region of the CP gene in the infection cycle of PSV. The significance of variation in the length of CP on aphid transmission also worth investigation. It should be noted that PSV, similar to other cucumoviruses, is aphid-borne and CP is a primary determinant of transmission [9, 27].

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