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Characterisation of black locust isolates of *Peanut stunt virus* (PSV) from the Pannon ecoregion show the frequent occurrence of the fourth taxonomic PSV subgroup

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Abstract Biological and molecular characterisation of ten *Peanut stunt virus* (PSV) isolates from *Robinia pseudoacacia* was carried out. The host range of these isolates was similar to that of the previously described PSV strains in most cases, but on *Pisum sativum* and *Lens culinaris* latent infection was induced. Variability in systemic symptoms was observed only on *Nicotiana glutinosa*. The partial RNA3 sequences were determined, including the carboxyl terminal region of the movement protein gene, the intergenic region, the entire coat protein gene and the 3′ untranslated region. Nucleotide sequence comparison of the coat protein genes showed 77.6–84.2% identity with most of the known PSV strains and 96.3–98.0% identity with PSV-Rp the typical member of subgroup IV. Phylogenetic analysis indicated the presence of the ancient homologous recombination in all of the examined black locust isolates and all the isolates were members of the fourth PSV subgroup. These results showed that the isolates of the fourth subgroup are widely distributed in black locust in this region.

Keywords *Peanut stunt virus* · PSV · Cucumovirus · Recombination · Legumes

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Peanut stunt virus (PSV) is an economically important pathogen of legumes worldwide, causing severe symptoms on alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), peanut (*Arachis hypogaea*), various types of clover (*Trifolium* spp.) and black locust (*Robinia pseudoacacia*) all over the world (Mink 1972) except Australia. Disease surveys revealed that black locust trees, a well established tree in central Europe, are widely infected by PSV and could act as a primary virus source for legumes and other susceptible plants.

PSV is a member of the genus Cucumovirus in the family Bromoviridae. Other members of the genus are *Tomato aspermy virus* (TAV) and the type member *Cucumber mosaic virus* (CMV) (Fauquet and Mayo 2001). The genome of PSV consists of three genomic RNAs with positive polarity, designated RNA1, RNA2 and RNA3, in order of decreasing size. RNA1 and RNA2 code for the viral components of the replicase complex. RNA2 also codes for a small protein called 2b, which is responsible for the suppression of post-transcriptional gene silencing and also functions in host-specific long-distance movement (Netsu et al.,

2008). This small protein overlaps with the carboxyl terminus of the 2a protein and is translated from the subgenomic RNA4A. RNA3 is dicistronic and encodes two proteins, the movement protein (3a protein, MP) and the coat protein (CP), which is translated from the subgenomic RNA4 (Hu et al. 1997; Hu and Ghabrial 1998; Palukaitis and Garcia-Arenal 2003).

PSV was first described in the USA in 1966 (PSV-E strain) (Miller and Troutman 1966) and then PSV-W was reported in 1969 (Mink et al. 1969). Initially, PSV strains were divided into two subgroups based on serology, competition hybridisation or sequence identity of RNA3: eastern (subgroup I) for PSV-E and related strains and western (subgroup II) for PSV-W (Hu et al. 1997). Later based on the complete primary sequence of the Chinese PSV-Mi isolate, the establishment of subgroup III was proposed (Xu et al. 1998; Yan et al. 2005). Recently, based on the nucleotide (nt) sequence of PSV-Rp, a fourth subgroup was proposed (Kiss et al. 2008). Nevertheless, numerous publications suggest that the phylogenetic relationships among PSV strains are more complex. Richter et al. (1987) suggesting six serogroups in Europe, and molecular hybridisation analysis showed that a black locust isolate of PSV (previously named *Robinia mosaic virus*, RoMV) cannot be classified in either subgroup I or subgroup II of PSV (Militao et al. 1998).

Beczner and Devergne (1979) characterised a PSV strain (PSV-Tp) isolated from *Trifolium pratense* in Hungary. This strain seemed to be more closely related to subgroup I than to the strains of subgroup II, but it had been identified as a new strain serologically-distinct from these subgroups (Devergne and Cardin 1976). A Polish strain, PSV-P isolated from yellow lupine (*Lupinus luteus*) was also identified, which is related, but not a typical member of subgroup I (Obrepalska-Steplovska et al. 2008a, b).

Previously we determined the complete nt sequence of a PSV strain (PSV-Rp) isolated from black locust in Hungary. Nt sequence comparison showed 74.1–84.6% identity with the known PSV strains. Recombination breakpoint analysis of PSV-Rp revealed two recombination points on RNA3. Based on these results the establishment of subgroup IV of PSV was proposed (Kiss et al. 2008). But still little is known about the diversity of PSV strains in Europe and the host range of subgroup IV is yet to be investigated. In this study we analysed ten additional PSV isolates (including PSV-Rp) originating from black locust, and the results of comparative studies of their host range, symptomatology and partial nt sequences of RNA3 (segment of MP, intergenic region (IR), CP gene and 3′ untranslated region (3′UTR)) are presented.

Ten PSV isolates were collected from black locust (*R. pseudoacacia*) showing severe mosaic and malformation symptoms (Fig. 1), between 2002 and 2008 from the Pannon ecoregion (Table 1). In natural infections of black locust, symptoms were visible only on few ends of branches of trees. *Chenopodium quinoa* plants were inoculated with extracts from the black locust leaves showing symptoms, and from single lesions obtained, *Nicotiana benthamiana* plants were infected. Symptoms induced by PSV isolates were compared on 14 plant species under greenhouse conditions. Three to eight plants of each species were sap-inoculated with infected *N. benthamiana* tissue

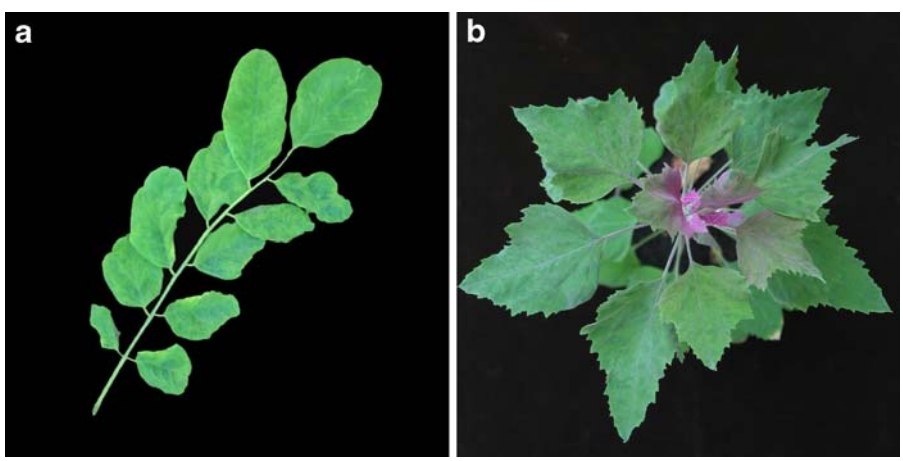

Fig. 1 Systemic symptoms of PSV-Rp2 on **a** *R. pseudoacacia* in natural infection, **b** on *C. amaranticolor*

Table 1 Location, data of collection and GeneBank accession numbers of the isolated PSV strains

Virus strain	Location of collection	Year of collection	Genomic component	Database accession number	References
PSV-Rp	Gödöllö, Hungary	2002	RNA3	AM905355	(Kiss et al. 2008)
PSV-B	Budapest, Hungary	2008	partial RNA3	FM992670	This study
PSV-Cs	Mezöcsát, Hungary	2008	partial RNA3	FM992665	This study
PSV-F	Füzesgyarmat, Hungary	2008	partial RNA3	FM992666	This study
PSV-Ljb	Ljubljana, Slovenia	2008	partial RNA3	FM992667	This study
PSV-Rp2	Gödöllö, Hungary	2007	partial RNA3	AM980675	This study
PSV-Sz	Szeghalom, Hungary	2008	partial RNA3	FM992671	This study
PSV-T1	Tihany, Hungary	2008	partial RNA3	FM992669	This study
PSV-T2	Tihany, Hungary	2008	partial RNA3	FM992668	This study
PSV-Tev	Tevel, Hungary	2008	partial RNA3	FM992672	This study

ground in 0.02 M potassium phosphate buffer, pH 7.0. The experiments were monitored for up to 1 month for symptom development. Symptomless plants were tested by Northern blot analysis (Sambrook et al. 1989) to determine latent infections. Total RNA was isolated from infected *N. benthamiana* plants, using the method described by White and Kaper (1989). RNA was dissolved in 30 µl sterile water; 2 µg of it was used for cDNA synthesis and 3 µg for Northern blot analysis. The reverse transcription was performed with M-MuLV RT (Fermentas) according to the manufacturer's instructions. cDNA fragments, covering the IR, entire CP gene, 3′UTR and partial MP gene of PSV RNA3 were amplified by PCR using Taq DNA polymerase (Fermentas) and universal PSV primers (UniPSV5′end: 5′ CARAARGCGATTGGTAGTGRG 3′; UniPSV3′end 5′ GGCTGCAGTGGTCTCCTAT GGAGCCCTCATAG 3′). The PCR products were purified and cloned using the pGEM-T-Easy Kit (Promega) and *Escherichia coli* DH5α (Invitrogen). Recombinant plasmids were isolated. The clones were selected by EcoRI digestion. Selected clones were sequenced from both directions using the universal M13 and M13 reverse primers. The nt and deduced amino acid (aa) sequences were analysed using the Emboss-Align (Rice et al. 2000) and Clustal X (using default parameters) programmes (Thompson et al. 1997). These programmes were also used for neighbour-joining analysis and genetic distance determination. Bootstrap analysis consisted of 1000 replications.

All the isolates of PSV were very similar based on their host range and symptomatology (Table 2). These isolates induced local chlorotic lesions, systemic mosaic and leaf malformation on *Chenopodium amaranticolor* (Fig. 1). In experimentally infected black locust (*R. pseudoacacia*) systemic mild mosaic symptoms were induced (Fig. 1), but 4 weeks after inoculations of cotyledons, the plants became symptomless as determined by Northern hybridisation (data not shown). PSV isolates caused latent infections in *P. sativum* and *Lens culinaris* while *Cucumis sativus* and *Lycopersicon esculentum* proved non-hosts of these isolates according to the Northern hybridisation (data not shown). Striking differences were observed in the reaction of *Nicotiana glutinosa*. PSV-Rp2, PSV-Sz, PSV-F and PSV-Ljb isolates induced systemic mosaic and ringspots (Table 2), while the other isolates did not induce even local symptoms on this host. The virus was detectable in the inoculated, but not in the non-inoculated leaves of *N. glutinosa* by Northern hybridisation (data not shown).

The complete nt sequence of PSV-Rp was determined earlier (Kiss et al. 2008). Sequence identities of the PSV-Rp and other PSV and cucumovirus strains were described (Kiss et al. 2008). The partial RNA3s of the other nine isolates were cloned and the nt sequences were determined. This size of the amplified region was between 1342 and 1351 nt (except PSV-Cs (1542 nt) which contained a 193 nt duplication in the 3′UTR). This fragment contained the previously determined recombination points of RNA3 (Kiss et al. 2008) and the complete CP gene was present. The nt sequence data was submitted to GenBank and the accession numbers are presented in Table 1. The determined nt sequences were compared with the PSV CP gene nt sequences

Table 2 Host range and symptomathology of the PSV isolates

Plants	Symptoms									
	PSV-B	PSV-Cs	PSV-F	PSV-Ljb	PSV-Rp	PSV-Rp2	PSV-Sz	PSV-T1	PSV-T2	PSV-Tev
Arachis hypogea	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu, LR	0/Mo, Stu
Chenopodium amaranticolor	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma	LLc/Mo, Ma
Cucumis sativus cv. Delicates	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −
Lens culinaris cv. Éva	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +
Lycopersicon esculentum cv. Kecskeméti jub.	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −	0/0, −
Medicago sativa	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo
Nicotiana benthamiana	0/Mo, Stu	0/Mo	0/Mo	0/Mo	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo	0/Mo, Stu
N. clevelandii	0/Mo, Stu	0/Mo, Stu	0/Mo	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu	0/Mo, Stu
N. glutinosa	0,+/0, −	0,+/0,−	0,+/Mo, RS	0,+/Mo, RS	0,+/0, −	0,+/Mo, RS	0,+/Mo, RS	0,+/0, −	0,+/0, −	0,+/0, −
Phaseolus vulgaris cv. Babylon	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma	0/Mo, Ma
Pisum sativum cv. Rajnai törpe	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +	0/0, +
Robinia pseudoacacia	0/Mo	LLc/Mo	0/Mo	0/Mo	LLc/RS, Mo	LLc/RS, Mo	LLc/Mo	LLc/Mo	LLc/RS, Mo	0/Mo
Vigna sinensis cv. Black eye	LLc/Mo	LLc/Mo	LLc/Mo	LLc/Mo	LLc/Mo	LLc/Mo	LLc/Mo	LLc/Mo, Ma	LLc/Mo	LLc/Mo
Zinnia elegans	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo	0/Mo

[a] Inoculated leaves/uninoculated leaves, *0* no symptom, *LL$_c$* chlorotic local lesion, *LR* leaf roll *Ma* malformation, *Mo* mottle or mosaic, *Stu* stunt, *RS* ring spot, +/− Northern hybridisation results of symptomless plants (positive/negative)

available in GenBank and with other representatives of the genus Cucumovirus, namely CMV-Fny, CMV-Trk7 and TAV-KC. The nt sequence identities for the CP gene among PSV isolates originating from black locust were between 96.3 and 98.0% and at the aa level sequence identities were between 98.1 and 100%. The nt sequence identity with other well-known PSV strains and with some cucumoviruses were as follows: PSV-ER (nt: 78.2–79.4%, aa: 73.2–75.0%), PSV-W (nt: 77.6–79.3%, aa: 70.5–72.8%), PSV-Mi (nt: 82.6–84.2%, aa: 85.3–86.6%), CMV-Trk7 (nt: 55.3–58.6%, aa: 49.1–50.0%), CMV-Fny (nt: 53.4–54.8%, aa: 47.5–49.3%), TAV-KC (nt: 65.2–67.5%, aa: 69.9–71.7%). Black locust isolates were quite different from other PSV isolates and with respect to identity they were fairly similar to each other. The great majority of the aa differences located between the aa 78–109 of the CP gene, and most of them were present only in one or two isolates (data not shown). The degree of identity among black locust isolates was much higher than observed between the CP gene of strains ER and J (91%), which have been classified as members of the subgroup I (Hu et al. 1997). Phylogenetic analysis was carried out with the complete nt sequence of the cloned region containing recombination breakpoints around nt 1,199 and 1,873 (numbers refer to the PSV-Rp RNA3) and with the segment located between the recombination points separately, including the different PSV strains, representatives of various cucumoviruses and *Brome*

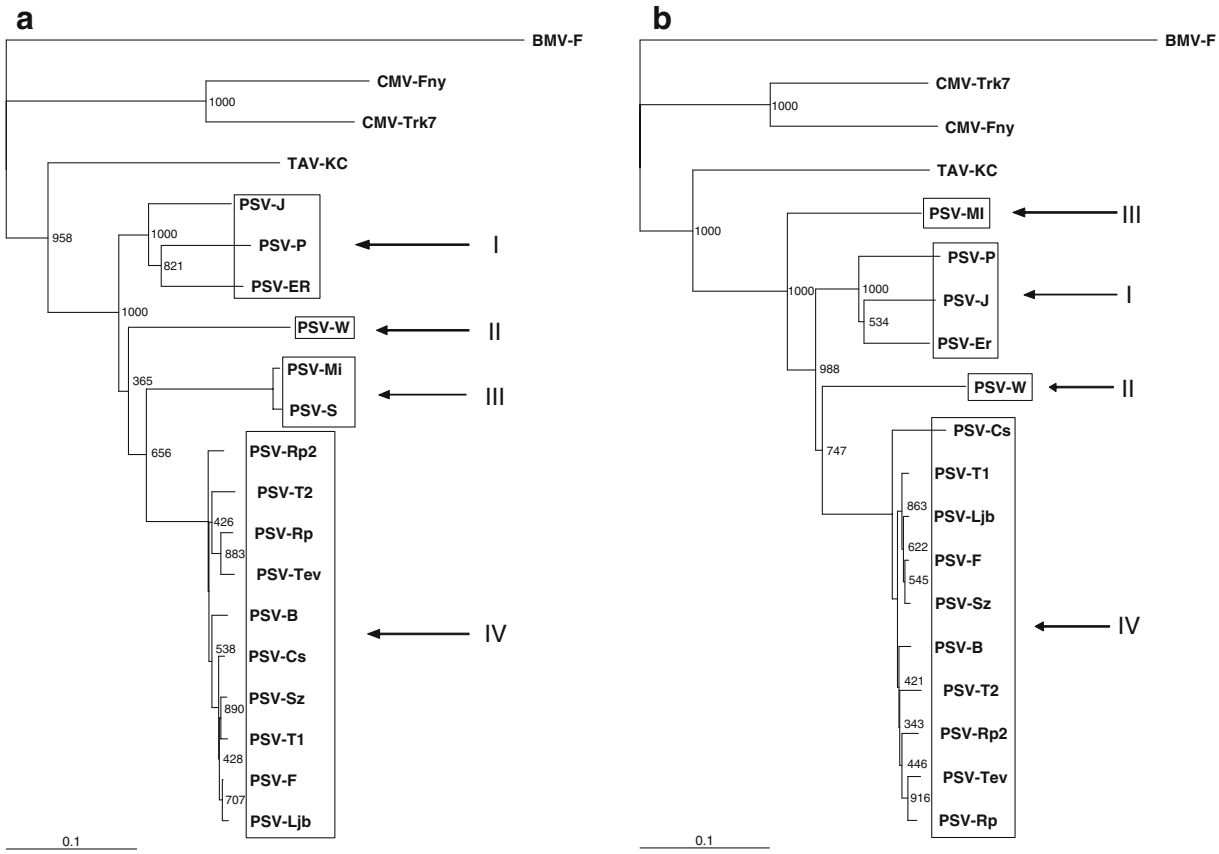

Fig. 2 Phylogenetic analysis of the different regions of cucumovirus RNA3: **a** coding nt sequence of the recombinant stretch, **b** coding nt sequence of partial MP gene, IR, CP gene and 3′UTR and containing the previously determined recombinant stretch. Numbers at nodes indicate the percent occurrence of nodes in 1000 bootstrap re-sampling. The bar on both tree corresponds to a 10% difference. Tetragones and roman numerals indicate PSV subgroups. BMV-F (DQ530425) was used as an outgroup. Nt sequences of previously determined strains were obtained from GeneBank. Accession numbers: PSV-ER: U15730, PSV-W: U31366, PSV-Mi: AY775057, PSV-P: EU570238, CMV-Fny: D10538, CMV-Trk7: L15336, TAV-KC: AJ237849

mosaic virus (BMV) as an outgroup. The neighbour-joining trees showed clearly that black locust strains had a closer relationship than the PSV-ER and -J strains (Fig. 2). In the case of the whole sequenced region of RNA3, black locust isolates clustered with PSV-W, but in the case of the recombinant stretch it clustered with PSV-Mi. These results support the presence of an ancient recombination event in the evolutionary history of RNA3 (Kiss et al. 2008).

Within the Cucumovirus genus, PSV is genetically the most diverse, followed by CMV and TAV. Despite wide genetic diversity, in contrast to the wide host range of CMV, PSV has a relatively narrow host range. On the basis of host reactions, nt sequence analysis and phylogenetic analysis, the ten black locust isolates of PSV were found to be distinct from the previously determined three PSV subgroups. All the investigated PSV isolates caused similar symptoms on most of the examined host plants. They infected *C. amaranticolor* systemically and induced mosaic like the Chinese strains but by contrast caused severe malformation on upper leaves (Fig. 1). It is known, that many PSV strains like the Hungarian PSV-Tp isolated from *T. pratense* infected *C. amaranticolor* only locally (Mink et al. 1969; Beczner and Devergne 1979). Based on these results *C. amaranticolor* could be a separating host of these isolates. PSV strains isolated from *R. pseudoacacia* induced latent infections on *L. culinaris* and *P. sativum*. In the systemic symptoms of the examined isolates a clear difference was observed on *N. glutinosa*, since only four of the isolates induced systemic infection on this host, while the other isolates were detected only in the inoculated leaves. In the case of cucumoviruses the CP (Taliansky and Garcia-Arenal 1995; Salánki et al. 1997) and the 2a protein (Du et al. 2008) are shown to have a role in long-distance movement. In this case there was no consistent difference in the CP aa sequences between the isolates infecting *N. glutinosa* systemically or locally. Therefore we suspect that the 2a protein has a central role in this respect, and the molecular background of this feature is under investigation.

Hajimorad et al. (1999) proposed that the cucumoviruses classified within the same subgroup should have at least 90% nt sequence identity for the whole genome, while the nt sequence identity between the different subgroups should be <80%. The black locust isolates of PSV were clearly distinct from other PSV strains and showed very high nt sequence and aa identity with each other and constituted a consistent subgroup. In this subgroup the sequence identities are much higher compared to the members of subgroup I, namely PSV-ER and PSV-J (91%). The recombinant PSV strain is widely distributed and the molecularly characterised isolate originated from black locust solely in the Pannon ecoregion up to the present.

Although numerous authors suggest that phylogenetic relationships among PSV strains are fairly complex in Europe (Beczner and Devergne 1979; Richter et al. 1987; Palukaitis and Garcia-Arenal 2003; Obrepalska-Steplovska et al. 2008), the phylogenetic analysis of the partial sequences of PSV isolates from black locust show a distinct fourth, coherent subgroup of PSV. The characterisation of central European isolates from other natural hosts of PSV is required for a clearer picture of evolution and genetic diversity of PSV strains.

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