Genetic Variability of the Czech *Pea enation mosaic virus* Isolates

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Abstract

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The *Pea enation mosaic virus* is an example of symbiogenesis of taxonomically unrelated PEMV-1 and PEMV-2 viruses. Partial RNA sequences, PEMV-1 coat protein, PEMV-2 movement protein gene and satellite RNA from seventeen isolates were compared with the aim of enlarging the knowledge of PEMV variability. The isolates showed genetic variability based on the nucleotide sequences in both RNA1 and RNA2, with 98–94% identity within coat protein gene sequences, and 95–96% identity within the movement protein. The phylogenetic analyses showed different evolution of both symbiotic viruses and differences in the European isolates. Most of the differences seen were synonymous without changes in the structure of proteins. The analysis of satellite RNA positive isolates implies a possible correlation between the structure of coat protein gene and presence of RNA3.

Keywords: coat protein; PEMV-1; PEMV-2; phylogenetic analysis; satellite RNA

The *Pea enation mosaic virus* (PEMV) is a virus infecting legume crops such as pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and faba bean (*Vicia faba*). It causes various symptoms of infection, including the typical vein enations, plant growth reduction and pod and seed deformities that can lead to significant yield losses. The virus is transmitted in a circulative manner by aphids, mainly by the pea aphid (*Acyrthosyphon pisum*), and by mechanical inoculation, but the seed transmission was not observed (Timmermann-Vaughan *et al*. 2009). The *Pea enation mosaic virus* represents a unique example of virus speciation. The previously described composite bipartite character of the virus genome (Demler *et al*. 1993), i.e. the presence of different taxonomically unrelated single stranded RNAs, is now understood as a unique example of symbiogenesis of PEMV-1 and PEMV-2 viruses (Roossinck 2005). *Pea enation mosaic virus 1* (PEMV-1), represented by the larger RNA1 molecule is the only member of the genus *Enamovirus* (family *Luteoviridae*), *Pea enation mosaic virus 2* (PEMV-2) represented by the smaller RNA2 molecule belongs to the genus *Umbravirus* (family unassigned). Both RNAs are coding the RNA-dependent RNA polymerase and they have replicative autonomy but their interaction is necessary for virus spreading and systemic infection. PEMV-1 encodes the coat protein which encapsidates the RNA1 and RNA2 in separate particles; and it encodes the protein involved in aphid transmission (AT) of PEMV. On the other hand, PEMV-2 is encoding the movement protein which is necessary for PEMV-1 systemic invasion in the plant (Demler *et al.* 1993, 1994a, 1997; Skaf *et al*. 1997). In addition to RNA1 and RNA2 molecules, PEMV isolates can contain a third, small satellite RNA (Demler & de Zoeten 1991). The satellite RNA is dependent on both helper viruses, the presence of PEMV-1 is necessary for the virus encapsidation and transmission and PEMV-2 for the virus replication (Demler *et al.* 1993, 1994b).

As the knowledge of PEMV variability is quite limited, mostly restricted to original American isolates, the analyses of European isolates with the focus on differences in PEMV-1 coat protein gene and satellite RNA are discussed in this study.

MATERIAL AND METHODS

Plant material and virus isolate. The plants showing a different range of pea enation mosaic disease were collected in the main Czech pea growing areas – Smržice, Šumperk, Chlumec and Troubsko during the pea virus disease survey. Seventeen virus isolates were transmitted by mechanical inoculation (Šafářová *et al.* 2008) onto the pea plants of cv. Raman and long-term maintained in the growth chamber at 22/18°C day/night temperatures under a periodic 16/8-h day/night cycle. The presence of PEMV-1 as well as the co-infection by PSbMV was confirmed by DAS-ELISA test (Löewe Biochemica, Sauerlach, Germany) according to the manufacturer's instructions.

RNA isolation and reverse transcription. Total RNA was extracted from 100 mg of fresh leaves using the TRI-reagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. The isolated RNA was subjected to reverse transcription using random primers (Promega, Madison, USA). The RT was carried out in two steps in a volume of 40 µl. In the first step 5 µl of RNA was mixed with 0.5µM of random primers and deionized water, and the mixture in a volume of 15 µl was denatured at 70°C for 5 min and chilled on ice. In the second step, AMV-RT buffer, 0.4µM of dNTPs, 20 U RNasin RNase inhibitor and 5 U AMV reverse transcriptase (all Promega) and deionized water were added to the mixture. The reverse transcription was run at 37°C for 60 min. RT products were immediately stored at –20°C.

PCR. Each of the virus RNAs was detected under different conditions using the specific detection primer pairs (Table 1). The reaction mix consisted of 5 µl cDNA, GoTaq (1×) buffer, 1.5mM $MgCl_2$, 0.625mM dNTPs, 1 U GoTaq DNA polymerase (all Promega), 0.5 µmol/l up and low primer and deionized water to the total volume of 20 µl. The PEMV-1 primers were designed to amplify the fragment covering the coat protein gene of PEMV-1, the primer pair was PERNA1up/PERNA1low. The PEMV-2 was detected using the primer pair PERNA2low/PERNA2up covering the fragment of the movement protein gene. The presence of satellite RNA was detected by primer pair PESATup/PESATlow. All primers were designed using the PrimerSelect software (Lasergene, DNAStar Inc., Madison, USA) The PCR conditions were pre-denaturation at 94°C for 2 min, 40 cycles of amplification (94°C for 30 s, annealing temperature depending on the primers for 30 s, and 72°C for 30 s), and final extension at 72°C for 5 min. 3 µl of PCR products were analysed by 2% agarose gel electrophoresis in a standard manner.

Cloning and sequencing. The PCR products were purified using a Gel Extraction Kit (Qiagen, Venlo, Netherlands), cloned into a pGEM-T plasmid and propagated in JM109 cells (Promega). Three clones of each isolate were sequenced using a BigDye v. 3.1 Terminator Sequencing Kit and an ABI PRISM 3130 sequencer (both Applied Biosystems, Foster City, USA). The fragments were assembled into final contigs by the SeqMan program (Lasergene package, DNAStar Inc.).

Phylogenetic analysis. Sequence identity was confirmed by identity screening of GenBank database using BLAST (Altschul *et al*. 1990). The corresponding homologous sequences were aligned by ClustalW algorithm and multiple sequence alignments of nucleotide and deduced amino acid sequences were used for the analysis of variability and the construction of a phylogenetic tree using the neighbour-joining method. The final trees were visualized by Tree Explorer software. All analyses were done using the Mega v. 5.21 software (Tamura *et al.* 2011)

RESULTS AND DISCUSSION

The occurrence of pea enation mosaic disease has been repeatedly confirmed in the most important pea growing areas in the Czech Republic (Piáková *et al.* 2006). To characterize its biological and genetic status a set of 17 infected plants has been investigated. The majority of the plants showed typical symptoms of the *Pea enation mosaic virus* infection as chlorotic leaf mosaics that later turned into translucent areas (so called windows), and vein enations developing on the lower side of leaves. In three cases no symptoms of the *Pea enation mosaic virus* infection were observed. The presence of PEMV-1 was detected in all seventeen cases by DAS-ELISA test. The screening for other pea viruses showed the presence of mixed infections with the *Pea seed-borne mosaic virus*. In these cases the severe leaf mosaics and stunting were observed.

To clarify the *Pea enation mosaic virus* genetic status, the presence and/or absence of three different RNAs was evaluated. The presence of PEMV-1 and PEMV-2 was confirmed in all analysed isolates, the fragments of the expected size 646 bp and 269 bp were amplified in RT-PCR with the newly designed specific PERNA1up/PERNA1low for PEMV-1 and PERNA2up/PERNA2low for PEMV-2 primer pairs

Table 1. List of primers used for detection of *Pea enation mosaic virus (*PEMV) RNAs

Primer	Sequence (5'-3')	Annealing temperature (°C)	Product length (bp)
PERNA1up	CCT CCA ATA AGC GAT GCC GAC TA	59	646
PERNA1low	GGG TGG AGG TGG TGG TGG TT		
PERNA2up	ACG GCG AGG TGA CAA TAA AAG AAC	58	269
PERNA2low	GAG GTG GGG GAG GAT GCT GTC		
PESATup	TCC CCG TGC GAC CCT TCC TA	54	314
PESATlow	TTA CAG CCG CTA CCA CCT CAG TCG		

(Table 1). No nonspecific products were detected in any of PEMV DAS-ELISA positive or negative samples. In three RNA samples (PE9, PE178 and PE329) isolated from the plants not showing the symptoms of pea enation mosaic disease the RT-PCR with PESATup/PESATlow allowed detecting the presence of the third RNA molecule, satellite RNA. The satellite RNA was detected only in samples which were isolated from plants not showing the disease symptoms.

The RT-PCR fragments of PEMV-1 coat protein gene, PEMV-2 movement protein and satellite RNA obtained were cloned and sequenced. The classification was confirmed by the comparison with *Pea enation mosaic virus* sequences available in GenBank using the BLAST algorithm. The coat protein gene sequences showed 98–94% identity with the PEMV-1 sequences. The movement protein sequences showed 96–95% identity with the PEMV-2 sequences. The satellite RNA fragments showed 98% identity with the isolate WSF (Acc. No. U03564.1), the only available sequence of PEMV satellite RNA, three Czech isolates differed in the presence of five informative sites (Figure 3). It was demonstrated earlier that PEMV exists in the plants as a mixture of aphid transmissible and non-transmissible isolates (Demler *et al*. 1997) but no difference in the obtained nucleotide sequences per PEMV RNA fragment and isolate was detected in our study.

The phylogenetic analysis of nucleotide and deduced amino acid sequences revealed a different status of PEMV-1 and PEMV-2. The nucleotide sequences of the full coat protein gene showed higher variability than amino acid sequences. The isolates were dis-

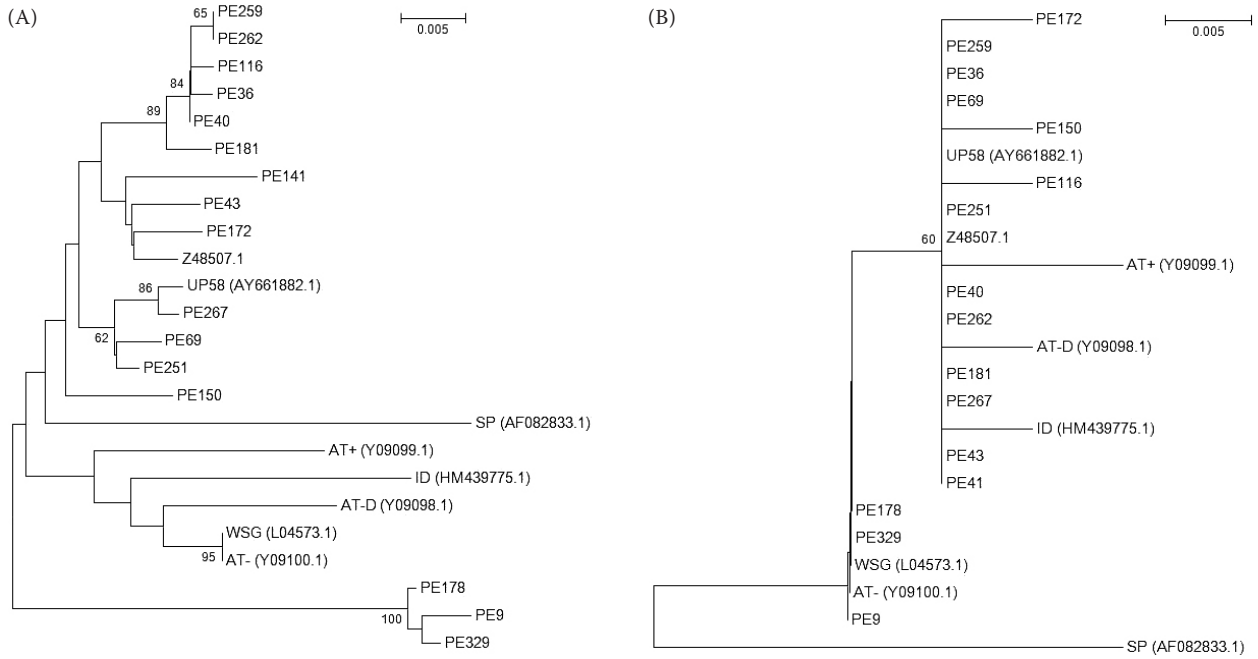

Figure 1. Unrooted dendrogram of PEMV-1 isolates constructed based on the nucleotide sequence (A) and deduced amino acid sequence (B) of coat protein gene; the scale bar represents 0.005 nucleotide substitutions per site; only the bootstrap values > 60% are shown

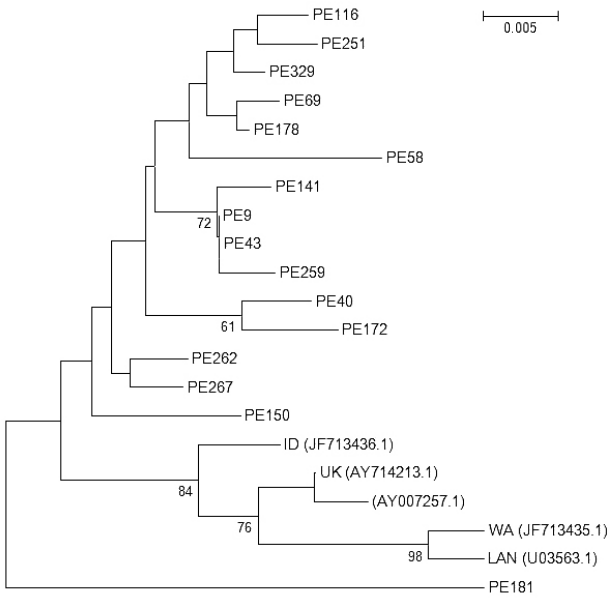

Figure 2. Unrooted dendrogram of PEMV-2 isolates constructed based on the partial movement protein gene; the scale bar represents 0.005 nucleotide substitutions per site; only the bootstrap values > 60% are shown

tributed among more or less significant five clusters, while the Czech isolates together with German isolate (Acc. No. Z48507.1) formed four clusters but different from the other sequences of *Pea enation mosaic virus* 1 described (Figure 1A). The American isolates grouped in the sixth distinct branch. The detected genetic variability confirms the differences between the European and non-European isolates predicted earlier by Saulgueiro and Hull (1999) and can be connected with different virus evolution in this area. The analysis of deduced amino acid sequences showed the functional reliability of this variation. Most of the detected nucleotide changes were synonymous. The amino acid sequences showed high homogeneity and the isolates formed only two branches (Figure 1B). One branch comprised the majority of the isolates irrespective of their geographic origin and/or expected aphid transmission status. The type and frequency of non-synonymous mutations in the coat protein support the hypothesis that the existence of the 54 kDa protein produced by aphid transmissible isolates cannot be connected with the variability in this ORF (Demler *et al.* 1997; Saulgueiro & Hull 1999).

A specific situation was noticed in the three isolates, PE9, PE178 and PE329, which are together with the WSG isolate the only available PEMV-symbiogenic complexes with the confirmed presence of satellite RNA. The isolates constituted one significant branch in a dendrogram constructed based on the nucleotide sequences and they also showed a distant position together with WSG and AT- isolates (Figure 1B) in the amino acid analysis that was represented by the presence of the parsimony informative site K/R_6. The satellite RNA does not show homology with the other PEMV viruses, but it was demonstrated that it is dependent on PEMV-1 for encapsidation and transmission (Demler *et al*. 1994b). Both sequence analyses imply the existence of a correlation between the structure of the coat protein gene and the presence of satellite RNA. This hypothesis needs to be supported by analyses of a broader spectrum of satellite RNA+ PEMV isolates.

The phylogenetic analyses of PEMV-2 nucleotide and deduced amino acid sequences yielded a different tree topology to the previous ones. The Czech isolates showed a low variability in the analysed fragment of 269 bp in length (position 2855-3123 in Acc. No. JF713435.1). Except for the isolate PE181, they constituted one branch, the isolates with the

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WSG      1 TCCCCGTGCGACCCTTCCTACGAAGCAGGTGGAAGATCCTTCCGTAGGTTGAAACTAAGAGGAACCTAGTGTGACTATGG
PE9      1 ..........................................................................T.....
PE178    1 ................................................................................
PE329    1 ................................................................................

WSG     81 TAGCTACAGTGTTGGCTGTTGTTGCCCCTCGCTCCCCCCCACTAGGGTGGTGCAGTCGTGGTCACATTGAGAGAGCAAAC
PE9     81 ..............................................A.........................G......
PE178   81 ............................A...................A.........................G......
PE329   81 ..............................................A.........................G......

WSG    161 CGAGCTTGGATTAAAGTTAGCGAACGCCTCCCCTGAGCCTCCAATGCTGTTACGATGAGGCAGATGGCATTGTGCAAGGG
PE9    161 ..................................A.....................................TT....
PE178  161 ........................................................................TT....
PE329  161 ........................................................................TT....

WSG    241 GGTAGTGGTTGGTAATGGTTCACACTGACTGCCGACGTAAACGTCAGTGTCGACTGAGGTGGTAGCGGCTGTAA
PE9    241 ............................................................................
PE178  241 ............................G...............................................
PE329  241 ............................G...............................................
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Figure 3. Partial nucleotide sequence alignment of PEMV satellite RNA (position according to U03564.1$^{260–573}$, WSG isolate)

present satellite RNA were distributed in two branches without evident difference (Figure 2). All GenBank available PEMV-2 sequences were distributed in the second significant tree branch. The analysis of amino acid sequences confirmed the synonymous status of the majority of substitutions; the only significantly distinct branch comprised the isolates WA and LAN (data not shown). The different topology of the nucleotide tree is in agreement with the different taxonomic status of PEMV-2 and its genetic independence on PEMV-1 (Demler *et al*. 1993).

In summary, the Czech PEMV isolates showed nucleotide variability but most of the detected mutations were synonymous. Phylogenetic analyses imply a correlation between the structure of the coat protein gene and the presence/absence of satellite RNA.

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