GENETIC AND BIOLOGICAL DIVERSITY OF THE PEA SEED-BORNE MOSAIC VIRUS ISOLATES OCCURRING IN CZECH REPUBLIC

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Summary. – Eight isolates of the Pea seed-borne mosaic virus (PSbMV) from the Czech Republic were studied regarding their biological and molecular characteristics. Molecular characterization using RT-PCR was done on the 5'(Nter)NIb-CP-UTR3' region amplified using universal CPUP/P9502 primer pair and the newly designed PSB8812/PSB944, and PSB8800/PSB9440 primer pairs, respectively. Sequential and phylogenetic analysis of CP-UTR3' region from all isolates showed that the available Czech and GenBank PSbMV isolates were distributed into 4 clusters in agreement with their diversification and according to their biological characteristics (i.e. pathotype). The molecular data were confirmed by biological testing on different pea cultivars. The Czech isolates were distributed into two pathotypes, the P-1 (7 isolates) and P-4 (1 isolate).

Key words: Pea seed-borne mosaic virus; pathotypes; phylogenetic analysis; sequencing

Introduction

PSbMV is a member of the family *Potyviridae*. This virus causes stunting, downward rolling of leaflets, swelling of leaf veins, and delayed flowering of most cultivars of *Pisum sativum*. Besides the pea, other economically important host plants for PSbMV are lentil, chickpea, and broad bean. The virus is transmitted in a non-persistent manner by aphids and vertically through seeds (Hampton and Mink, 1975). PSbMV was discovered in former Czechoslovakia (Musil, 1966), but probably is distributed worldwide due to a dissemination from germplasm collection, breeding lines, and commercial cultivar seeds (Grünwald *et al*., 2004). The resistance of pea to transmission of PSbMV was described and four recessive resistance genes were identified allowing the identification and grouping of viral isolates into the pathotypes. Standardized pathotypization was developed based on the reaction of the differential host pea genotypes (Alconero *et al*., 1986; Hjulsager *et al*., 2002). The gene *sbm-1* confers resistance to the pathotype P-1 represented by the pea isolates US or DPD1; *sbm-2* and *sbm-3* genes confer resistance to the pathotype P-2 represented by the lentil isolate L1 and *sbm-4* gene confers resistance to the pathotype P-4 represented by the pea isolates NY and S6 (Provvidenti and Alconero, 1988; Johansen *et al*., 1991). The broad bean isolate NEP-1 is considered as the pathotype P-3 based on its biological characterization (Hjulsager *et al*., 2002). The P-1 pathotype seems to be prevalent in Europe, North America, Australia, New Zealand, Pakistan and P-4 pathotype is found in Australia, North America, and Pakistan (Alconero *et al*., 1986; Timmerman *et al*., 1990; Johansen *et al*., 1991; Ali and Randles, 1997). The geographical distribution of P2 and P3 pathotypes is not known. Both pathotypes are represented by unique members, e.g. P-3 is represented by NEP-1 isolated from the seed material originating from Nepal and P-2 is represented by isolate L1 from the germplasm accession (Alconero *et al*., 1986; Hjulsager *et al*., 2002). Since the PSbMV is

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Abbreviations: AMV = Alfalfa mosaic virus; BCMV = Bean common mosaic virus; PEMV-1 = Pea enation mosaic virus 1; PSbMV = Pea seed-borne mosaic virus; CP = coat protein; NIb = nuclear inclusion b protein; UTR = untranslated region

transmissible by seeds, this propagation material represents an important phytosanitary risk.

This study reports molecular and biological characterization of the PSbMV isolates obtained in Czech Republic with recognition of their pathotypes.

Materials and Methods

Virus isolates. The PSbMV isolates were obtained from naturally-infected pea plants from three different localities Smržice, Šumperk, and Troubsko (Table 1) during the year 2003–2005. The presence of the PSbMV in plants as well as the co-infection with Pea enation mosaic virus 1 (PEMV-1), Alfalfa mosaic virus (AMV), and Bean common mosaic virus (BCMV) was confirmed by DAS-ELISA (Loewe Biochemica). The PSbMV isolates were mechanically transmitted and maintained on the pea cultivar Merkur.

RT-PCR. Total RNA was extracted from 50 mg of fresh pea leaves using RNeasy Plant Mini Kit (Qiagen). The reverse transcription was carried-out in two steps, in total reaction volume of 40 µl. The primer annealing mixture contained 5 µl of total RNA, 0.4 µmol/l oligo (T)18 primer and deionized water in total volume of 15 µl. The mixture was denatured at 70°C for 5 mins and chilled on ice. The annealed mixture was completed with AMV RT 5x buffer, 0.4 mmol/l dNTPs, 20 U RNasin® RNase Inhibitor (Promega), 5 U AMV reverse transcriptase (Promega), and deionized water. The RT was run at 42°C for 60 mins.

The two overlapping fragments covering the 5'(Nter) NIb-CP-UTR3' region (1108 bp and 1115 bp lengths, respectively) were obtained with the three different primer pair combinations. The first fragment was amplified using the potyviral universal primer pair P9502 and CPUP (van der Vlugt and Bouwen, 1997). For amplification of the second part of the region, the two combinations of newly designed primers, PSB8812 (5'-TTGAGAAATA CACGGAAGC-3') and PSB9440 (5'-CATTATCTGTCTGAA AGTTGG-3'); or primer PSB8800 (5'-ACAAAGTGAATTAGA AAGGTA-3') and PSB9440, were used. The PCR conditions involved pre-denaturation 94°C for 2 mins, 40 cycles of amplification (94°C for 60 secs, 52°C for 60 secs, and 72°C for 60 secs), and a final extension at 72°C for 10 mins. The PCR amplifications were carried out in mixture that consisted of 5 µl cDNA, RedTaq (1x) buffer, 1.5 mmol/l $MgCl_2$, 0.625 mmol/l dNTPs, 0.5 µmol/l sense and antisense primer, 1 U RedTaq Polymerase (Sigma), and deionized water to the total volume of 25 µl. The 5 µl of PCR products were analyzed by 2% agarose gel electrophoresis in a standard manner.

Cloning and sequencing. The PCR products were purified using a Gel extraction kit (Qiagen), cloned into a pGEM-T plasmid and propagated in JM109 cells (Promega). Three clones of each isolate were sequenced using a BigDye v. 1.1 sequencing terminator kit and an ABI PRISM 3130 sequencer (both Applied Biosystems). The fragments were assembled into the final contigs with the SeqMan program (Lasergene package, DNASTAR, Inc.).

Phylogenetic analysis. Sequences were checked for homologous sequences in GenBank, using the BLAST program (Altschul *et al*., 1990) and were aligned with corresponding sequences of other available PSbMV isolates. 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 a neighbor-joining method ClustalW. The bootstrap option was run with 1000 re-samplings. The tree was visualized by TreeView program v. 1.6.1 (Page, 1996).

Biological testing. For the pathotype determination, the pea cultivars Fjord, No. 8720 (PI 193586), Bonneville (PI 471187), and Sankia (PI 269774) were used (Hjulsager *et al*., 2002). Selected PSbMV isolates were mechanically transmitted on the plants. For mechanical transmission, 1 g of infected leaf tissue was grinded with 4 ml of 10 mmol/l phosphate buffer (pH 8.0), supplemented with 1% activated charcoal and Celite. The obtained homogenate was rubbed onto two bottom leaves of an experimental plant 10 days after planting. The symptoms were observed at one-week interval and the systemic infection was confirmed with DAS-ELISA.

Results and Discussion

The occurrence of PSbMV on the pea plants was found in each of the studied growing locations Smržice, Šumperk, and Troubsko in Czech Republic. The infected pea plants manifested symptoms of leaf rolling, stunting, mild mosaic,

Table 1. Characterization of PSbMV isolates according to their infectivity on the pea cultivars

Isolate designation	Locality	Susceptibility of pea cultivars to infection				Pathotype
		Fjord	No. 8720 PI 193586	Bonneville PI 471187	Sankia PI 269774	
PSB58CZ	Smržice	S	R	S	S	P-4
PSB117CZ	Smržice	S	R	S	R	P-1
PSB118CZ	Smržice	S	R	S	R	P-1
PSB141CZ	Smržice	S	R	S	R	P-1
PSB178CZ	Šumperk	S	R	S	R	P-1
PSB329CZ	Šumperk	S	R	S	R	P-1
PSBDCZ	Troubsko	S	R	S	R	P-1
PSBECZ	Troubsko	S	R	S	R	P-1

S = susceptible, R = resistant.

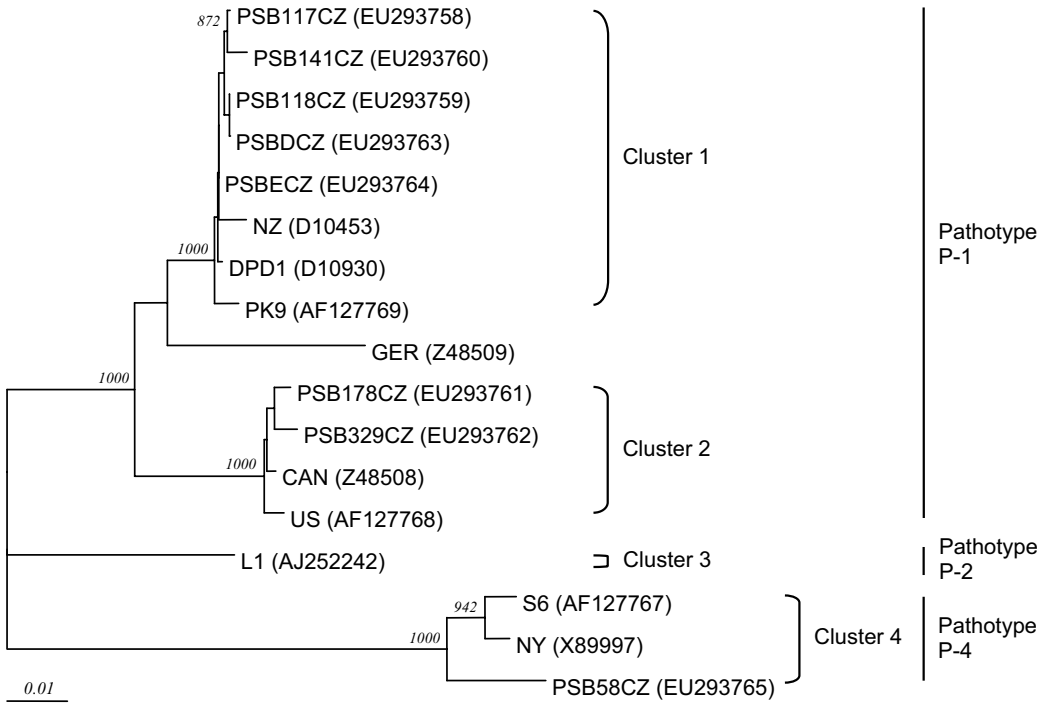

Fig. 1

Phylogenetic tree of PSbMV isolates reconstructed from the 1106 bp of the region 5'(Nter)NIb-CP-UTR3'

The scale bar represents 0.01 nucleotide substitutions per site. Only the bootstrap values >70% are shown.

and vein clearing. The infection with PSbMV was confirmed by DAS-ELISA in all samples. In some cases, mixed infections of PSbMV with PEMV-1 were found.

The eight PSbMV isolates were included in this study. All of them were easily mechanically transmissible and were maintained on the pea cultivar Merkur. The first symptoms of down-rolling leaves were noticed 12 days after inoculation. During the following two weeks the symptoms of vein clearing and leaf yellow mosaic developed. The growth reduction, previously described for NY isolate (Johansen *et al*., 1996), was typical for the infection by the PSB58CZ isolate.

The 5'(Nter)NIb-CP-UTR3' region of analyzed isolates was amplified using RT-PCR and all fragments were cloned and sequenced. The amplification of the first fragment, using the universal CPUP/P9502 primer pair was successful in all cases. On the other hand, the amplification of the beginning of the CP gene using the newly designed primer pair PSB8812/PSB9440 gave the expected product at a length of 629 bp only for 7 of the 8 isolates. Because the PCR amplification was negative in the case of the isolate PSB58CZ, the successful amplification of a similar fragment of this isolate required to design another primer PSB8800. Afterwards, the amplicon of a length of 641 bp was obtained using the PSB8800/PSB9440 primer pair. The subsequent analysis of the obtained sequence showed that the isolate PSB58CZ lacked the appropriate priming site of primer PSB8812.

The full sequence of chosen segment was obtained for each isolate and their classification as PSbMV was confirmed by comparison with the PSbMV GenBank sequences using the BLAST algorithm. The sequences of 8 tested isolates were deposited in GenBank under Acc. Nos.: EU293758 (PSB117CZ), EU293759 (PSB118CZ), EU293760 (PSB141CZ), EU293761 (PSB178CZ), EU293762 (PSB329CZ), EU293763 (PSBDCZ), EU293764 (PSBECZ), and EU293765 (PSB58CZ).

Phylogenetic analysis based on the 1106 bp long nucleotide sequences of 5'(Nter)NIb-CP-UTR3'region (position 8814–9919 nt according to the isolate DPD1) showed distribution of the Czech isolates into 3 significantly distant clusters (Fig. 1). The five Czech isolates (PSB117CZ, PSB141CZ, PSB118CZ, PSBDCZ, and PSBECZ) clustered with the non-American isolates DPD1, GER, NZ and PK9 in cluster 1; the two Czech isolates (PSB178CZ and PSB329CZ) formed cluster 2 together with the North American isolates. The lentil isolate (L1) formed the separate cluster 3. Only one of the Czech isolates, PSB58CZ belonged to cluster 4 together with the isolates from Pakistan (NY) and Australia (S6). Analysis of the shorter sequence according to Andersen and Johansen

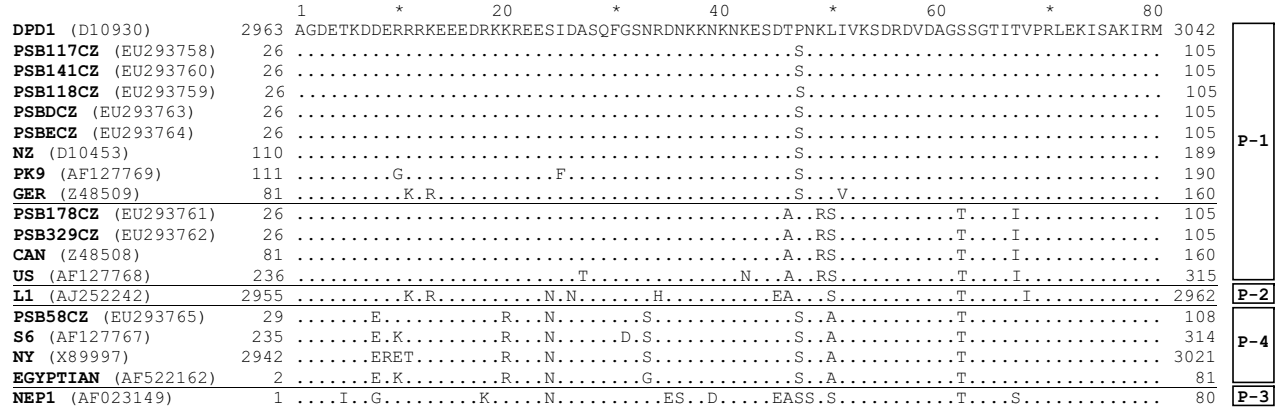

Fig. 2

Partial amino acids sequence alignment of PSbMV isolates

The first 80 deduced amino acids of the coat protein are shown (position aa 2693–3042 according to PSbMV isolate DPD1).

(1998), e.g. the variable beginning of the CP gene with a length of 418 bp (position 8889–9306 nt in the isolate DPD1) allowed the comparison of a larger number of isolates and gave the same topology of the phylogenetic tree with the same distribution of Czech isolates into clusters. The isolate NEP-1, that could have been included in the analysis this way, represented the fifth significant cluster (data not shown).

The analysis of the deduced amino acids sequences of the CP gene showed the presence of typical parsimony informative sites and also supported the previous diversification. Discrete clusters were characterized by the highly conserved amino acids L_{50}, S_{62} for cluster 1, R_{49}, I_{67} for cluster 2, N_{26}, H_{34} for cluster 3, and E_{8}, R_{20}, A_{50} for cluster 4 (Fig. 2). In all cases, the DAG motif in position 59–61 was conserved, in agreement with the previously described position (Timmerman *et al*., 1990).

The distribution of the isolates into the 4 clusters corresponded with the division of the PSbMV isolates into pathotypes according to their infection profile on *P. sativum* cultivars. All previously characterized type isolates of pathotypes P-1 (DPD1, US), P-2 (L1), P-3 (NEP1), and P-4 (NY, S6) are members of the formed branches (i.e. groups). Accordingly, the classification of our isolates into pathotypes P-1 and P-4 can be assumed from the biological testing performed on the pea cultivars Fjord, No. 8720 (PI 193586), Bonneville (PI 471187), and Sankia (PI 269774). We noted that the pathotype-specific infection confirmed the classification of the 7 Czech isolates PSbMV (PSB117CZ, PSB141CZ, PSB118CZ, PSBDCZ, PSBECZ, PSB178CZ, and PSB329CZ) as pathotype P-1 and one isolate (PSB58CZ) as pathotype P-4 (Table 1).

Summing up, the molecular analysis of 5'(Nter)NIb-CP-UTR3' region of 8 Czech PSbMV isolates in agreement with the biological testing demonstrated their distribution into two pathotypes P-1 and P-4. According to our information, the classification of the isolate PSB58CZ as the pathotype P-4 represented the first detection of the PSbMV isolate with this characteristic in Europe.

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