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Plum pox virus accumulates mutations in different genome parts during a long-term maintenance in *Prunus* host plants and passage in *Nicotiana benthamiana*

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Summary. – Plum pox virus (PPV) isolates of the strain PPV-M prevalently infect peaches under natural conditions in Middle Europe. Comparison of complete genome sequences obtained from subisolates of a PPV-M isolate maintained experimentally over a 6-year period in different *Prunus* host species and passaged in *Nicotiana benthamiana* was performed with the aim to highlight the mutations potentially connected with the virus-host adaptation. The results showed that the lowest number of non-silent mutations was accumulated in PPV-M maintained in peach (original host species), approximately two times higher diversity was recorded in plum, apricot and *N. benthamiana*, indicating the genetic determination of the PPV host preference. The sequence variability of *Prunus* subisolates was distributed more or less evenly along the PPV genome and no amino acid motif could be outlined as responsible for the host adaptation. In *N. benthamiana* the mutations were accumulated notably in the P1 and P3 genes indicating their non-essentiality in the infection of this experimental host plant.

Keywords: sharka; host adaptation; mutation; evolution

The plum pox virus (PPV) causing sharka disease of stone fruit trees is currently divided into eight molecularly distinct strains (chronologically D, M, EA, C, Rec, T, W, and CR) and the knowledge on the PPV variability further widens with accumulated sequence data (Šubr and Glasa, 2013). So far, only for PPV-C the strain affiliation has been found strictly connected with the natural host specificity (cherries). Other PPV strains, however, show also some preference for their natural host plant species, e. g. plums were found massively infected by PPV-D and PPV-Rec, and peaches by PPV-M under field conditions (Šubr and Glasa, 2013). Genetic predestination of such behaviour may be anticipated. Similarly to other potyviruses, the PPV genome codes for nine non-structural proteins and the capsid protein (CP) derived proteolytically from a polyprotein, and at least one polypeptide arising by frameshift translation in the P3 gene region, called PIPO (Wei *et al*., 2010). Here we present a study of the PPV host-dependent genome variability. Complete sequence analyses of several subisolates of the PPV-M isolate maintained for six years in three *Prunus* species were performed to discover potential spontaneous PPV genome adaptations to particular host plants.

The PPV-M isolate VAR-2, maintained on GF305 in the insect-proof conditions (Glasa *et al*., 1997) was chip-budded on 1-year old peach (*Prunus persica*), apricot (*P. armeniaca*) and plum (*P. domestica*) trees in summer 2006. The trees were maintained under field conditions and grown in close proximity to each other. In 2012 (after 6 years) the genomic sequence of PPV from each source was estimated. The sequence data were obtained from directly sequenced overlapping PCR products after reverse transcription of total RNA isolated from symptomatic leaves (Glasa *et al*., 2004). The sequences of subisolates were compared to the VAR-2 sequence determined from the original source (lyophylised

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Abbreviations: aa = amino acid; CP = capsid protein; ncr = non-coding region; PPV = plum pox virus

in 1999) using MEGA5 software (Tamura *et al*., 2007). A full-length sequence from VAR-2 passaged two times in *Nicotiana benthamiana* was included for the comparison.

Obtained sequences were submitted to the GenBank database under Acc. Nos. HF585100-HF585104. The results of the multiple alignment comparison, showing the differences between the respective subisolates are summarized in the Table 1. Despite the absence of replication proofreading, the master sequences of RNA viruses tend to be quite stable due to selection process in their natural host species (Schneider and Roosinck, 2001). Therefore it was not surprising that the determined differences among the complete nucleotide (nt) sequences of VAR-2 subisolates were very low (26–31 nt corresponding to the variability 0.27–0.32%). When comparing the nt divergence for different genome parts, the highest variability level (about 2 %) was linked to the 3′ non-coding region (ncr). On the other hand, the CP gene remained well conserved, with no amino acid (aa) difference among *Prunus* VAR-2 subisolates. In the polyprotein-coding region the highest absolute number of nt differences were found in HC-pro and CI genes, however, their relative incidence did not exceed 0.5%. The PIPO region was 100% conserved in all subisolates. Comparison of VAR-2 subisolates with original VAR-2 sequence showed that the differences were most uniformly distributed in the genome of the apricot subisolate (seven genes with non-silent mutations); in the other subisolates the aa differences were found in 3–4 proteins. The ratio of non-silent/silent nt differences along the polyprotein-coding region was 0.15 (peach), 0.37 (plum), 0.45 (apricot), and 0.47 (*N. benthamiana*). Although the peach subisolate was most divergent at the nt level, it showed the lowest aa difference from the original VAR-2 sequence.

PPV-M tends to infect mainly peaches under field conditions (Pasquini and Barba, 1997). One can therefore expect that the long-term experimental maintenance in atypical host species could lead to selection and fixation of mutations overcoming this feature. PPV VAR-2 was originally isolated from peach (Glasa *et al*., 1997). Although no clear relation between the sequence and host adaptation was found in our experiment (i.e. no aa exchanges and only two silent nt exchanges in HC-pro correlated with the peach/non-peach origin of the subisolates), the sequence comparison showing two-times higher aa conservation in peach than in other *Prunus* species indicated the host preference could be genetically determined.

The VAR-2 subisolate from *N. benthamiana* differed from the *Prunus* subisolates by notable accumulation of mutations in the P1 (1.3% diversity) and P3 (1.1% diversity) genes, while the rest of the genome retained highly conserved in this host plant (Table 1). In the P1, the polymorphisms were found in the central part and the C-terminal protease domain (Adams *et al*., 2005), while in the P3 they were localised in the N-proximal part of the protein upstream the PIPO frameshift (Table 2). P1 probably cooperates with HC-pro in the inhibition of plant defense by post-transcriptional gene silencing (Valli *et al*., 2006). Other functions of both P1 and P3 proteins in the infection cycle remain still mysterious, although they have been shown to be pathogenic determinants in various potyvirus-host systems (Sáenz *et al*., 2000; Hjulsager *et al*., 2006). High interviral and low intraviral variability of these proteins allows us to presume their role in specific virus-plant interactions, determining the host range of particular potyviruses (Valli *et al*., 2007). *N. benthamiana* is known to potentially host a wide range of viruses and other pathogens. PPV infects this species

Table 1. Number of nucleotide (amino acid) differences between original VAR-2 sequence (HF585100) and the subisolates from various hosts in particular PPV genome regions

Genome region	Total nt (aa)	Δ plum HF585102	Δ apricot HF585103	Δ peach HF585104	Δ *N. b.* HF585101
5′ ncr	146	0	0	2	0
P1	924 (308)	1 (1)	1 (0)	1 (0)	11 (4)
HC-pro	1374 (458)	4 (0)	6 (1)	3 (0)	1 (0)
P3	1050 (350)	1 (1)	2 (1)	2 (2)	6 (4)
6K1	156 (52)	1 (0)	1 (1)	1 (1)	0 (0)
CI	1905 (635)	7 (2)	7 (2)	9 (1)	3 (0)
6K2	159 (53)	1 (0)	2 (1)	0 (0)	0 (0)
VpG	501 (167)	1 (0)	3 (2)	0 (0)	1 (0)
NIa-pro	807 (269)	4 (3)	0 (0)	1 (0)	0 (0)
NIb	1554 (518)	1 (0)	2 (1)	5 (0)	0 (0)
CP	993 (330)	0 (0)	1 (0)	2 (0)	1 (1)
3′ ncr	218	5	4	5	5
polyprotein	9784 (3140)	26 (7)	29 (9)	31 (4)	28 (9)

Table 2. Overview of the amino acid variability in the gene products of VAR-2 subisolates from various sources. Different residues as in the original VAR-2 sequence are typed in bold. The numbers correspond to the polyprotein aa positions. Nested variations are boxed.

	Protein/aa position																									
VAR-2	P1				HC	P3					6K1		CI					6K2	VPg		NIa-pro			NIb	CP	
subisolate	159	162	273	303	609	875	876	879	1079	1100	1124	1161	1263	1273	1279	1316	1680	1819	1877	1880	2132	2145	2182	2294	3137
Orig. (peach)	A	A	Q	S	T	F	D	K	I	S	N	I	L	V	T	I	T	N	A	V	V	K	P	K	V
Plum sub.	A	**T**	Q	S	T	F	D	K	I	**L**	N	I	L	**G**	**I**	I	T	N	A	V	**M**	**R**	**S**	K	V
Apricot sub.	A	A	Q	S	**A**	F	D	K	I	**L**	**S**	I	**V**	V	T	I	**A**	**T**	**P**	**G**	V	K	P	**Q**	V
Peach sub.	A	A	Q	S	T	F	D	K	**T**	**L**	N	**V**	L	V	T	**V**	T	N	A	V	V	K	P	K	V
N. benth. sub.	**V**	**T**	**H**	**N**	T	**L**	**V**	**Q**	I	**L**	N	I	L	V	T	I	T	N	A	V	V	K	P	K	**M**

systemically, spreads fast and multiplies massively in all plant tissues (Goodin *et al.*, 2008). Such a high susceptibility may indicate that specific interactions essential for infection of natural host species are not required in *N. benthamiana*. Consequently, non-silent mutations may occur in the viral genes responsible for such interactions.

The detected aa differences between VAR-2 subisolates are specified in the Table 2. Several polymorphisms were nested by two or three in particular sequences. Most exchanges referred to similar aa residues according to PAM250 similarity matrix (Altschul, 1991). Exchanges with most significant potential influence on the protein structure/function were the S/L dimorphism in P3 (not correlated to the peach/non-peach origin of subisolates) and two V/G dimorphisms found in different positions in the non-peach subisolates, namely in CI (plum) and VPg (apricot). When compared to other PPV strains, some correlation of aa sequences was found only for the A/T^{162} dimorphism in P1, where threonine was present in VAR-2 plum subisolate, as well as in sequences of PPV-D and PPV-Rec (data not shown), both infecting predominantly plums under field conditions in Middle Europe (Šubr and Glasa, 2013). A single aa exchange may influence the protein function resulting in substantial phenotype modification (Merour *et al.*, 2013; Nagyová *et al.*, 2012; Seo *et al.*, 2011; Šubr *et al.*, 2010). On the other hand, potyviral proteins are multifunctional and they cooperate in processes involved in virus-host interactions (Decroocq *et al.*, 2006), therefore the complex phenotype (e.g. host preference) is probably influenced by several viral genes.

The sequence data obtained from our experimental work did not allow pinpointing any aa motif along the PPV polyprotein which could be connected to different *Prunus* host origin of PPV subisolates. Direct sequencing of PCR products in our experiment enabled to obtain and analyze the prevalent (leader) sequences of the virus quasi-species populations from particular host plants. Whereas a relatively high PPV nt heterogeneity has been recorded in a single infected tree (Jridi *et al.*, 2006; Predajňa *et al.*, 2012), the possibility that some of minor virus forms enabled the efficient PPV infection of naturally atypical host species by a trans-complementation mechanism cannot be excluded. Relatively high mutation rates observed for P1 and P3 (but not the PIPO frameshift product) on the aa level compared to other viral genes during few passages in *N. benthamiana* might suggest a low essentiality (or non-essentiality) of these proteins in the infection of this experimental herbaceous host species.

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