

The 3'-proximal part of the Plum pox virus P1 gene determinates the symptom expression in two herbaceous host plants

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Abstract Three major strains of the Plum pox virus (PPV) are the most important in Europe: PPV-D, PPV-M, and PPV-Rec. By combining the genomes of two different strains of PPV (PPV-D with PPV-Rec; PPV-D with PPV-M), 20 inter-strain chimeric infectious clones (CICPPV) were constructed. Biological properties of CICPPV were tested by inoculating them on different herbaceous host species susceptible to PPV. Four of the seven species tested, exhibited visible symptoms. In *Nicotiana benthamiana* all CICPPV induced systemic mosaic and leaf malformation. *Pisum sativum* showed a broad range of symptom severity (systemic chlorotic and necrotic lesions) but neither qualitative nor quantitative aspects of symptomatology were related to a single PPV genome locus. *Nicotiana occidentalis* and *Nicandra physaloides* proved to be suitable for symptom-based differentiation. Depending on the virus strain/chimera, *N. occidentalis* showed two types of symptoms: mild systemic chlorotic spots or local necrotic lesions/systemic vein necroses. *N. physaloides* reacted to the PPV infection either symptomless or by local necrotic lesions. Our results demonstrated that the P1/HC-pro region of the PPV genome appears to be the determinant of the symptom manifestation in these host plants. In silico analysis mapped it to the 3'-proximal part of the P1 gene.

Keywords Sharka · Infectious cDNA ·
Inter-strain chimera · Pathotype determinant

Introduction

Plum pox virus (PPV) causes sharka, the most detrimental disease of the stone fruit trees. It occurs in Eurasia, Africa, and America [1, 2]. Although the natural host range of PPV is restricted to *Prunus* spp., several herbaceous species can be experimentally infected, mostly from the genus *Nicotiana* [3]. *N. benthamiana* or *N. clevelandii* are commonly used propagative host species of PPV.

PPV is a potyvirus with (+)ssRNA genome coding for a polyprotein, from which at least 10 viral proteins are released by proteolytic processing: P1, HC-pro, P3, 6K1, CI, 6K2, VPg, NIa-pro, NIb, and CP. Capsid protein (CP) is the only polypeptide creating the viral capsid (about 2,000 CP copies per virion). VPg is covalently bound to the 5' terminus of the genome. No other proteins are present in the viral particles. Most of the potyviral proteins are known to be multifunctional. They participate in viral genome replication, movement through the infected plant, suppression of host defense by gene silencing, or virus transmission among plants by vectors [4].

Infectious cDNA clones of RNA viruses provide an excellent tool for the research of viral gene functions and virus-host interactions. Constructed inter-strain or inter-virus chimeras enable to map the pathogenicity determinants in the viral genomes [5–8].

Seven PPV strains are currently recognized, from which PPV-M, PPV-D, and PPV-Rec have the most substantial impact in Europe [2]. Infectious cDNA clones of these PPV strains have been already prepared [9–12]. The recently discovered major strain, PPV-Rec, demonstrated for the first time the importance of homologous recombination in the PPV evolution [13]. Although the PPV-Rec genome consists of the parts with high similarity to PPV-D and PPV-M, respectively, some strain-specific mutations have

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been fixed which are conserved in PPV-Rec isolates [14]. Broad natural spread of PPV-Rec in several European countries and sporadically outside Europe [15, 16] demonstrates its high fitness in competition with other strains [17].

In this work, a set of inter-strain PPV-D/PPV-Rec and PP-D/PPV-M chimeras (chimeric infectious clones, CICPPV) is constructed and used to test their pathogenicity in herbaceous host plants to localize the genome parts responsible for differently expressed symptomatology.

Materials and methods

The infectious clone pIC-PPV (strain PPV-D) [10] was kindly obtained from Prof. J. A. García (CSIC, Madrid), the cDNA clone of the isolate SK68 (strain PPV-M; Acc. No. M92280) [18] from Prof. L. Palkovics (CU, Budapest). Total RNA was isolated from lyophilized PPV-infected plant tissue using NucleoSpin RNA Kit (Machery Nagel).

Reverse transcription was performed using random hexamer primers and AMV reverse transcriptase (Promega) [13]. cDNA of PPV isolates BOR-3 (strain PPV-Rec; Acc. No. AY028309) [19] and SK68 served as template for PCR using proof-reading DNA polymerase (La Taq, TaKaRa). The primers were designed according to the BOR-3 and SK68 sequences [14, 18] to amplify PPV genome fragments of appropriate length for the production of inter-strain chimeras. The sequence of all PCR products was verified. CICPPV combining pIC-PPV with BOR-3 or SK68 were constructed using FastDigest restriction endonucleases (Fermentas) as shown in Fig. 1. Most of the cloning sites were present in the original sequences of viral cDNA, in four cases (*SexAI* in BOR-3, *RsrII*, *SexAI* and, *SphI* in SK68) the restriction sites were introduced in the primers. The CICPPV were produced by cleaving the pIC-PPV by particular restriction enzymes followed by ligation of corresponding amplified genome fragment from BOR-3 or SK68 digested by the same enzymes. The plasmids were transformed and multiplied in *E. coli* JM109,

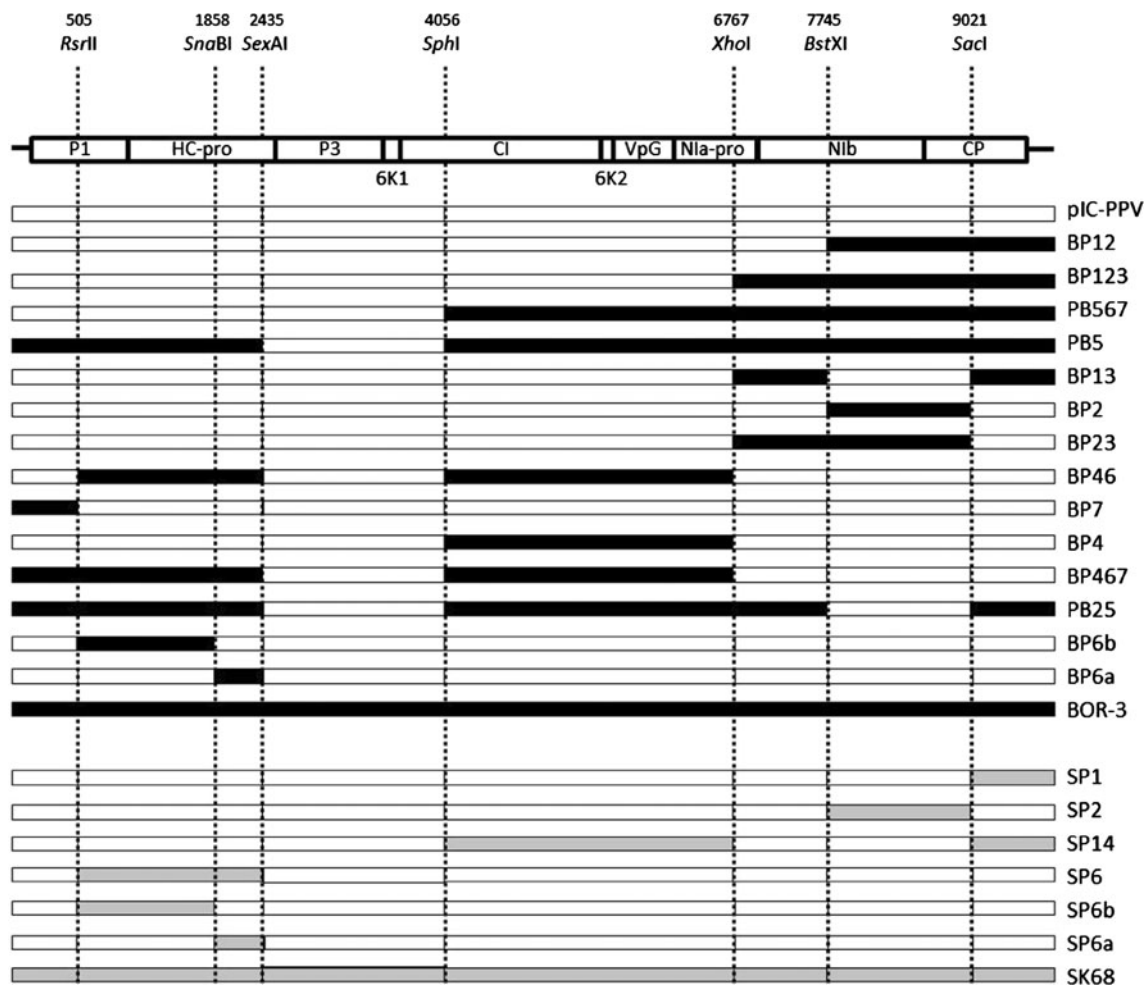


Fig. 1 Scheme of the PPV genome and the prepared inter-strain CICPPV. PPV-D sequences are represented by *empty rectangulars*, PPV-Rec by *black* and PPV-M by *gray rectangulars*. The position of restriction sites is numbered according to the BOR-3 sequence

purified using PureYield Plasmid Purification System (Promega), sequence verified, and used for plant transfection. Infectious cDNA clones were introduced into *N. benthamiana* plants by an airgun particle bombardment using tungsten microcarrier particles M-10 (Bio-Rad) as described [20]. A set of herbaceous plant species (each triplicated) was infected by the mechanical inoculation of crude sap from virus-positive *N. benthamiana*. Following species were used: *N. occidentalis*, *N. clevelandii*, *N. glutinosa*, *N. rustica*, *Nicandra physaloides*, and *Pisum sativum* cv. Colmo. The infection was evaluated by symptom observation and immunoblot analysis using polyclonal anti-PPV antibody [21] and alkaline phosphatase-conjugated anti-rabbit IgG antibody (Sigma). Virus antigen accumulation in plants was estimated by semi-quantitative DAS-ELISA using polyclonal antibody V.196 produced in INRA-EN-SAM (Montpellier) as previously described [22]. The samples were regarded positive if the absorbance value measured at 405 nm was at least three times higher than for the negative (healthy) control.

Results

A set of 20 PPV-D/PPV-Rec and PPV-D/PPV-M CICPPV was prepared (Fig. 1). To compare their biological properties to parental isolates, a panel of herbaceous experimental host plants was infected with each of the respective chimera. PPV was shown infectious for all species applied. Some of them, however, reacted symptomless or the displayed symptoms were very mild or ambiguously scored (in case of *N. clevelandii*, *N. glutinosa*, and *N. rustica*).

N. benthamiana showed similar symptoms for all tested PPV forms, namely systemic mosaic and leaf malformation (Fig. 2g). Only BOR-3 caused typical dark-green islands on the leaves, especially around the main veins in this plant species (Fig. 2f). Both types of symptoms, however, were not well mutually distinguishable and sometimes overlapped.

In the plants of *P. sativum* (cv. Colmo) pIC-PPV, six PPV-D/PPV-Rec and five PPV-D/PPV-M CICPPV induced sporadic necrotic lesions on the inoculated leaves (Fig. 2k) with no visible systemic symptoms or only few individual chlorotic spots on upper leaves 3 weeks post inoculation (Fig. 2l). BOR-3, SK68, and the rest of CICPPV were manifested by systemic chlorotic spots or mottling (Fig. 2m–p) with no symptoms on inoculated leaves. In addition, systemic necrotic lesions of various shape often occurred in later infection stage, 30–40 days post inoculation (Fig. 2q–s). The observed symptoms were not bound to one single genome locus. The necrotizing lesions were induced by CICPPV with at least a part of NIb and the P1/HC-pro region originated from different parental isolates (Table 1). The virus could not be detected in symptomless systemic leaves either

by immunoblotting, ELISA, or by RT-PCR, while the highest virus concentration estimated by semi-quantitative ELISA was found in leaves with intensive chlorotic mottling and necrotic lesions (data not shown).

The best candidates for PPV pathotyping were *Nicandra physaloides* (infection was mostly symptomless, but with several CICPPV, a hypersensitive response with local necrotic lesions was observed, Fig. 2i) and *N. occidentalis*, where either mild systemic chlorotic spots or local necrotic lesions and vein necrosis connected with leaf rolling occurred (Fig. 2b–d). Moreover, two different growth behaviors of infected *N. occidentalis* plants were observed. The habit of plants with rosette appearance of leaf lamina with delayed flowering (in 12 or more weeks of plant age) was for the purpose of this work named “habit A.” Other plants showed reduced leaf lamina with early flowering in plant age of 5–6 weeks (“habit B,” Fig. 2t). Habit A was typical for plants with weak leaf symptoms and habit B was connected with systemic vein necrosis and necrotic lesions on inoculated leaves.

The pathotypes observed for analyzed PPV isolates and CICPPV are summarized in the Table 1. In *N. physaloides*, necrotic lesions occurred on inoculated leaves after infection by the isolates BOR-3 (PPV-Rec) or SK68 (PPV-M). PPV was not able to move systemically through the plants, the virus was detected only in inoculated leaves. No symptoms were observed in plants infected by pIC-PPV (PPV-D), although the virus presence was proved by immunoblot in inoculated leaves. Most CICPPV induced symptomless infection as well. Three of the PPV-D/PPV-M CICPPV (SP1, SP2, SP14) did not multiply in inoculated leaves either. Five PPV-D/PPV-Rec CICPPV (PB5, BP46, BP467, PB25, and BP6b) and two PPV-D/PPV-M CICPPV (SP6 and SP6b) induced necrotic lesions on inoculated leaves of *N. physaloides*. In all of them, the *RsrII-SexAI* genome fragment (coding for C-terminal part of P1 and N-terminal part of HC-pro) was replaced in pIC-PPV by its homolog from BOR-3 or SK68.

The pathotyping on *N. occidentalis* similarly highlighted the influence of this genome part on the symptom expression. While pIC-PPV (PPV-D) caused mild mosaic symptoms in this host species related to plants in habit A, infection by BOR-3 (PPV-Rec) or SK68 (PPV-M) led to the change to habit B with local necrotic lesions on inoculated leaves and systemic vein necrosis. Habit B and systemic vein necrosis were observed also in plants infected by all CICPPV with P1/HC-pro region originating from these isolates: PB5, BP46, BP467, PB25, BP6b, SP6, and SP6b. Local necrotic lesions were induced also by most of them, with exception of BP467. On the other hand, CICPPV with the P1/HC-pro region from pIC-PPV resembled this isolate by inducing mild symptoms in *N. occidentalis* (Table 1).

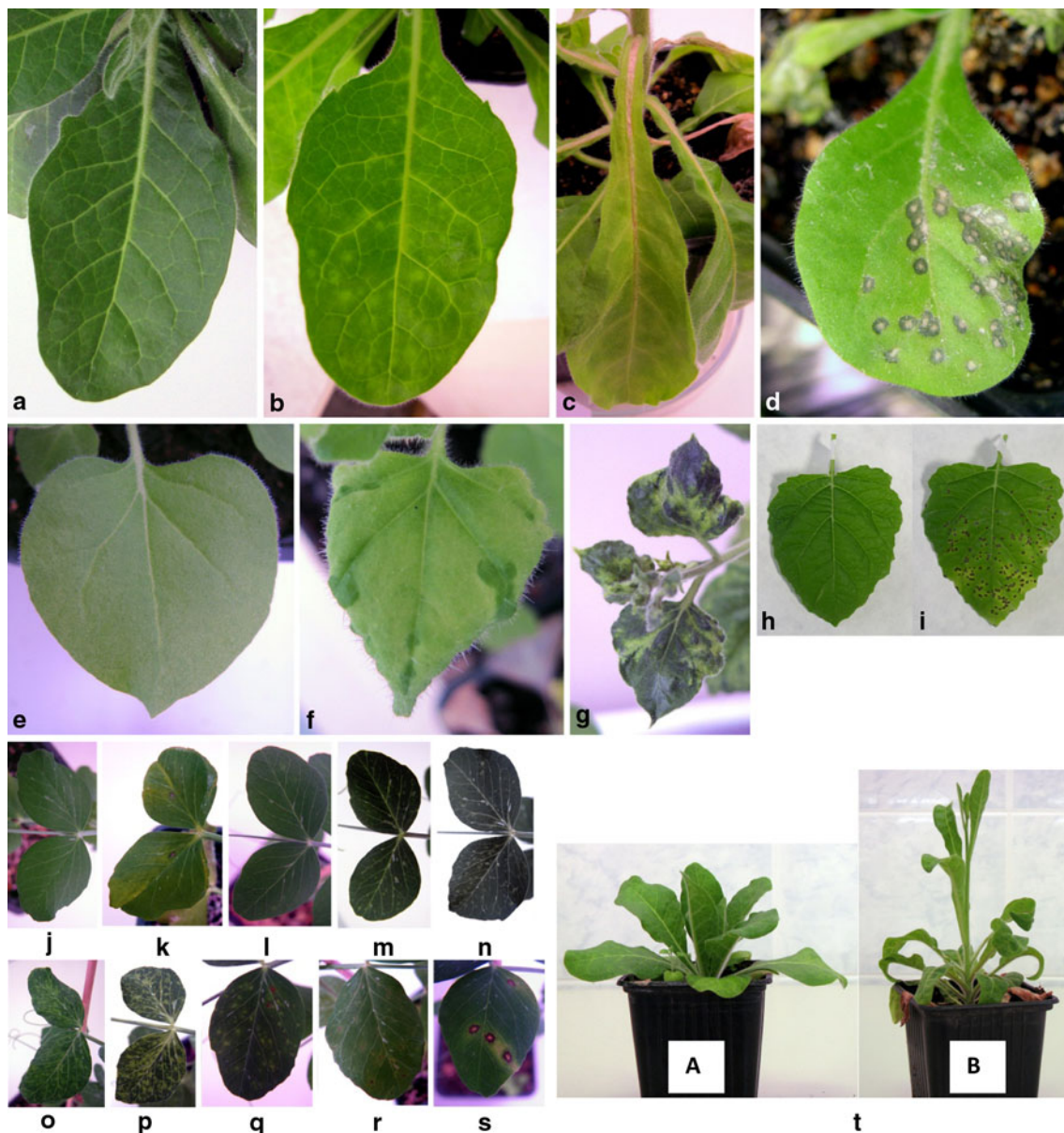


Fig. 2 Overview of the PPV symptomatology in herbaceous host plant species. *N. occidentalis*: healthy (a), mild chlorotic spots (b), vein necrosis (c), local necrotic lesions (d), *N. benthamiana*: healthy (e), vein-associated dark islands (f), mosaic/leaf distortion (g), *N. physaloides*: healthy (h), local necrotic lesions (i), *P. sativum*:

healthy (j), necrotic lesions on inoculated leaves (k), chlorotic spots of various intensity (l–p), systemic necrotic lesions of various intensity and shape (q–s), and growth habitus A and B of *N. occidentalis*, respectively (t)

Multiple alignment of the *RsrII-SnaBI* fragment-coding region of three parental PPV sequences (pIC-PPV, BOR-3, and SK68) showed that amino acid differences were located exclusively in the C-terminal part of P1, nucleotide variability of the relevant HC-pro gene part was silent (Fig. 3). Overall, four positions could be detected in P1 where amino acids differed for mild (pIC-PPV) and severe symptoms induced isolates (BOR-3 and SK68). These involved R/W¹³⁸, N/D/H²⁰⁰, V/I²⁵⁴, and I/V³⁰⁶.

Discussion

Prepared infectious clones of PPV-D and PPV-M have been applied in the research of virus-host interactions. The observed phenotypic changes included the host range, long-distance movement in the plant body or manifestation of specific symptoms in given host [6, 11, 23–25]. PPV multiplies generally well in various herbaceous hosts. Intensive passage of PPV isolates on herbaceous plants may even lead

Table 1 Symptoms caused by PPV chimeras

PPV strain (chimera)	Symptom type ^a		
	<i>N. occidentalis</i>	<i>N. physaloides</i>	<i>P. sativum</i>
pIC-PPV	0/MCS (A)	0/–	lnl/cl
BP12	0/MCS (A)	0/–	0/CNL
BP123	0/MCS (A)	0/–	0/CNL
PB567	0/MCS (A)	0/–	lnl/cnl
PB5	LNL/VN (B)	LNL/–	0/CL
BP13	0/MCS (A)	0/–	0/CNL
BP2	0/MCS (A)	0/–	lnl/cl
BP23	0/MCS (A)	0/–	0/CNL
BP46	LNL/VN (B)	LNL/–	lnl/cnl
BP7	0/MCS (A)	0/–	lnl/0
BP4	0/MCS (A)	0/–	lnl/cl
BP467	0/VN (B)	LNL/–	lnl/cnl
PB25	LNL/VN (B)	LNL/–	0/CL
BP6b	LNL/VN (B)	LNL/–	0/CL
BP6a	0/MCS (A)	0/–	0/CL
BOR-3	LNL/VN (B)	LNL/–	0/CL
SP1	0/MCS (A)	–/–	lnl/0
SP2	0/MCS (A)	–/–	0/CNL
SP14	0/MCS (A)	–/–	lnl/0
SP6	LNL/VN (B)	LNL/–	lnl/cnl
SP6a	0/MCS (A)	0/–	lnl/cl
SP6b	LNL/VN (B)	LNL/–	lnl/0
SK68	LNL/VN (B)	LNL/–	0/CL

0 Symptomless; MCS mild chlorotic spots; VN vein necrosis; LNL, lnl local necrotic lesions; CNL, cnl chlorotic/necrotic lesions; CL, cl chlorotic lesions (weak symptoms by lower-case font); and – no infection detected by immunological methods

^a Local/systemic symptoms (growth habit A or B see the text and Fig. 2v)

to the lack of ability to infect *Prunus* plants [6]. Repeated mechanical passages may also result in the selection of aphid non-transmissible PPV forms [26, 27]. Such accelerated evolution could disvalue the biological experiments made in these artificial conditions. On the other hand, woody host plants are generally difficult to manage, they require a prolonged evaluation time and sometimes could provide incorrect results, due to irregular virus distribution in single plants. Moreover, the PPV strains seem to be more or less adapted to different *Prunus* species [28] which may distort the obtained data. For example, Dallot et al. [6] mapped the PPV-D-*Prunus* host compatibility to the locus P3/6K1 in plum, but they were not able to localize it clearly when peach host was used. This might be related to the natural multi-genic host preference of PPV-D for plum and/or of complementing PPV-M for peach.

Recently, the complete nucleotide sequence of the PPV isolate BOR-3 (representing the PPV-Rec strain broadly

spread in central and south-eastern Europe) has been determined [13, 14]. As a consequence of homologous recombination, the 3' portion of PPV-Rec genome (3' end of NIb, whole CP gene, and 3'-NCR) shares high percentage of identity with PPV-M, while the rest (major part of the genome) resembles that of the PPV-D isolates. PPV-Rec is characterized by several conserved biochemical and biological properties. These include typical double-band form of CP in SDS-PAGE (due to different posttranslational modifications) mapped to the single amino acid position 66 in the CP [29], very mild symptoms (or symptomless infection) in GF305 peach seedlings [17], and a strong host preference (infecting almost exclusively plum trees and absent on peach under natural field conditions) [13]. Genetic mapping of specific PPV-Rec properties is a current challenge. Our strategy of CICPPV preparation relied on mutual exchanges of segments along the whole PPV genome across three major PPV strains. Their unmodified sequences were used, and only few silent nucleotide changes were introduced where the appropriate restriction sites were not present in viral cDNA.

We were able to infect all seven tested plant species with PPV and CICPPV. In *N. benthamiana* the symptoms developed by all parental isolates and chimeras could not be well mutually distinguished because of their similarity and generation of intermediate forms. The symptom intensity in *N. clevelandii*, *N. glutinosa*, and *N. rustica* was very low, and the infection was mainly symptomless. Therefore, these species were unsuitable for the evaluation of PPV symptom behavior.

Several distinguishable symptom types were observed in *N. occidentalis*. The replacement of the 5'-proximal genome fragment coding for 452 amino acids was responsible for the change of symptomatology in this species. Hypersensitive reaction of *N. physaloides* was mapped to the same region involving parts of P1 and HC-pro genes. These potyviral genes have been already pinpointed to be involved in virus-host interactions. P1 is the most variable potyviral protein with both recombination and gene duplication occurring in its evolutionary history, which contributed to widening the host species range [30]. RNA-binding activity of P1 has been shown [31]. Interaction of N-terminal P1 part with the host chloroplast Rieske Fe/S protein has been demonstrated and inclusion of such interaction in the formation of chlorotic symptoms was hypothesized [32]. Salvador et al. [33] showed that P1 was an important (although not exclusive) factor of potyvirus host specificity. Chimeric PPV with exchanged P1 from the Tobacco vein mottling virus was able to infect herbaceous hosts common for both parental viruses (despite extended sequence and length differences between both P1 genes), but not a *Prunus* host of PPV [33]. P1 is believed to enhance the PTGS inhibition by HC-pro and to be an accessory factor for genome

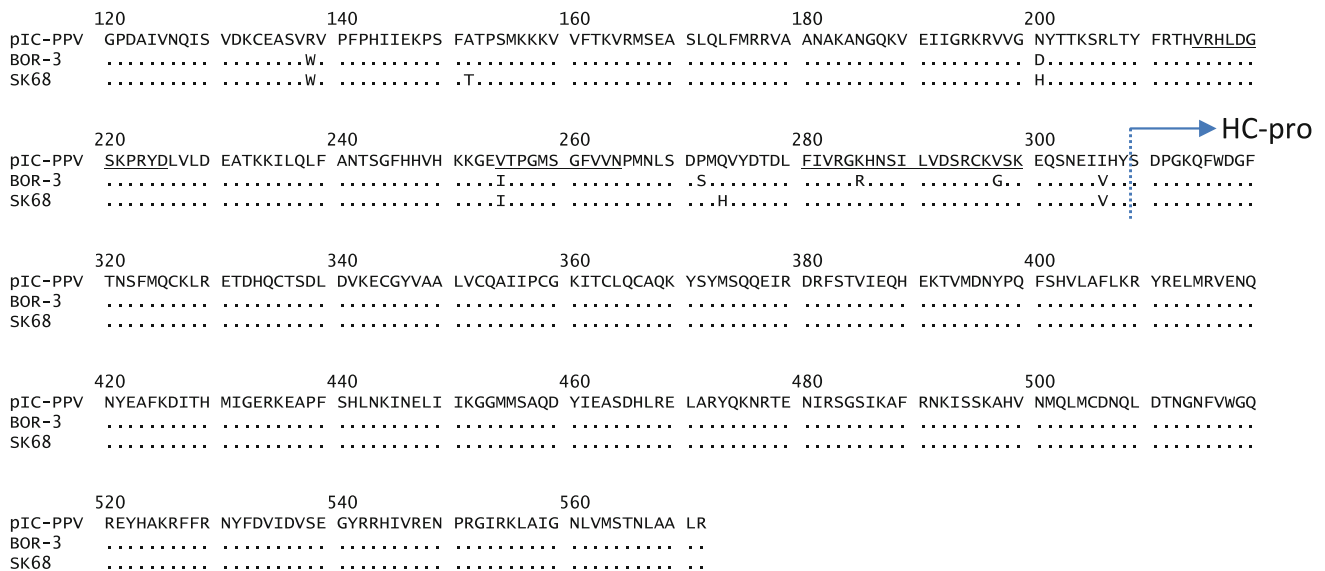


Fig. 3 Alignment of the amino acid sequences derived from the *RsrII-SnaBI* genome fragment of three PPV isolates. Numbering of residues from the beginning of the BOR-3 polyprotein (AY028309).

Identical amino acids are represented by dots. P1 protease motifs [30] are *underlined*, the cleavage site between P1 and HC-pro is indicated by a *dotted line and arrow*

amplification [34, 35]. Tobacco etch virus P1/HC-pro could complement the inability of PPV systemic spread in tobacco plants which has been given in connection with their PTGS suppression [36]. Interactions of HC-pro with several host plant proteins have been demonstrated in vitro and in vivo, including Ca^{2+} -binding calreticulin, chloroplast ferredoxin, chloroplast division-related factor NtMinD, and a novel RING finger protein [37–40]. All of them could be connected with symptomatology of particular infections, e.g., due to chloroplast injury or influence on the calcium signaling pathways. Two separate amino acids (but neither of them alone) in Clover yellow vein virus HC-pro have been mapped to cause necrotic symptoms expression in broad bean related to enhanced virus-induced gene silencing [41]. A single amino acid replacement in the PPV-M HC-pro has been shown responsible for substantially different severity of symptoms evoked in *N. occidentalis*. Subpopulations of the PPV isolate PS caused mild mottling or local necrotic lesions and vein necroses depending on Gly or Ser in the HC-pro position 232, respectively [24].

All three parental isolates from our experiments share 100% amino acid identity in the HC-pro part potentially contributing to their different symptomatology, and they contain Gly in this position (540 according to numbering from the polyprotein beginning, Fig. 3) indicating that HC-pro was not responsible for the observed symptom differences. Most of the amino acid variability among the three parental isolates was found close to the C-terminus of P1 protein known to host the proteolytic domain. However, the catalytic triad (H²¹⁶, D²²⁵, and S²⁵⁹) and the surrounding motifs [30, 42] were well conserved in all three

isolates (Fig. 3). Two of the four observed point heterogeneities correlating with the symptomatology in *N. occidentalis* (R/W¹³⁸ and N/D/H²⁰⁰) lay upstream the protease motifs, two others were localized either in the proteolytic domain (V/I²⁵⁴) or in the protease recognition motif close to the cleavage site between P1 and HC-pro (I/V³⁰⁶). Although the Val-Ile variability probably does not influence the protein function because of high similarity and mutual interchangeability of these amino acids, we currently cannot speculate if the observed phenotype differences reflect the proteolytic activity or rather another P1 function. It is notable that PPV-Rec amino acid sequence of this genome part is rather related to PPV-M than to PPV-D reflecting probably more recent recombination event hypothesized by Glasa et al. [43]. Consequently, the observed symptomatology of PPV-Rec in *N. occidentalis* resembled that of PPV-M.

While the symptomatology in *N. phyaloides* and in *N. occidentalis* was connected with the same genomic region, the situation was different in *P. sativum*. PPV induced in pea a systemic chlorotic spotting of different severity (from sporadic individual chlorotic lesions to intensive mottling). Some lesions became necrotic with time. Plants showing hypersensitive reaction on inoculated leaves were systemically symptomless or showed very weak symptoms. The relative virus amount in systemic leaves proved by ELISA correlated well with the intensity of symptoms, but not with the symptom type (only chlorotic or chlorotic + necrotic lesions) in agreement with the results of Saenz et al. [11]. While these authors mapped the induction of systemic necrotic lesions in *P. sativum* to

the P3/6K1 genomic region, either local or systemic symptoms could not be clearly connected with any viral protein in our hands. Different PPV isolates used in both studies (PS vs. SK68 and BOR-3) could cause this distinction. Cooperative involvement of Nib and P1/HC-pro could be outlined, indicating role of virus replication in the induction of necrotic phenotype. The symptoms in *P. sativum* reflected at least partially also the ability of PPV systemic movement. Involvement of CP, as well as 6K2 and VPg in long-distance movement of potyviruses has been shown [44–46]. Continual variability of the systemic infection intensity was observed in our experiments rather than clear ability/inability of the long-distance movement. Therefore, we could not outline any gene influence on the PPV systemic movement in *P. sativum*.

The observed symptomatology may be different in each virus-host systems despite identical functions of individual viral proteins in particular hosts. Also some action at the RNA level cannot be excluded, moreover, unknown out-of-polyprotein-ORF polypeptides may be produced during infection like the recently discovered PIPO protein [47]. Most of the observable phenotypes (symptoms and host range) are probably dependent on a complex of virus-host interactions, where several or all viral genes could be involved in some way. It is not surprising therefore that various parts of the potyviral genome have been shown to be included in host specificity or symptom manifestation depending on the model used. Our results confirmed the role of PPV P1 in virus-host interactions resulting in various pathotypes and demonstrated a different relative importance of particular PPV genes for symptom manifestation in different herbaceous host plant species. The behavior of prepared CICPPV in *Prunus* spp. will be the object of further studies especially regarding mapping the host preferences of PPV strains.

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