Arch Virol
DOI 10.1007/s00705-010-0677-1

BRIEF REPORT

A single amino acid mutation alters the capsid protein electrophoretic double-band phenotype of the *Plum pox virus* strain PPV-Rec

Z. W. Šubr · M. Kamencayová · S. Nováková · A. Nagyová · J. Nosek · M. Glasa

Received: 20 January 2010 / Accepted: 26 March 2010

Abstract *Plum pox virus* (PPV) isolates differ by their capsid protein (CP) mobility in SDS-PAGE. These electrophoretic phenotypes are likely to result from post-translational modifications of the CP. We demonstrated that the CP mobility was solely determined by the CP N-terminal region. Sequence comparison pinpointed a possible role of mutations at position 66 in determining the CP phenotype of PPV-Rec isolates. Site-directed mutagenesis of a chimeric clone demonstrated that Gly(66) in the CP resulted in the double-band phenotype, while Arg(66) led to a single-band CP pattern, possibly by preventing the phosphorylation of a nearby Ser residue by steric hindrance.

Plum pox virus (PPV), a member of the genus *Potyvirus*, causes sharka, the most important disease of stone fruit trees worldwide [1]. The extensive recent accumulation of sequence data for PPV isolates of various geographical origin has demonstrated that PPV heterogeneity is significantly higher than previously thought. Involvement of homologous recombination in the evolutionary history of PPV has also been demonstrated [2, 3], and seven strains of PPV are now recognized: PPV-D, PPV-M, PPV-EA, PPV-C, PPV-Rec, PPV-W and PPV-T [4, 5].

The capsid protein (CP) of PPV isolates has been known to show variability in electrophoretic mobility in denaturing polyacrylamide gel electrophoresis (SDS-PAGE), with the CP of PPV-M migrating more slowly than that of PPV-D [6, 7]. PPV-Rec originates from a homologous recombination event between PPV-M and PPV-D in the NIb gene, and its CP resembles that of PPV-M [3]. However, nearly all of the approximately 300 PPV-Rec isolates analysed to date in our laboratory (Šubr and Glasa, unpublished) showed a typical double-band pattern in SDS-PAGE, with one band migrating more slowly and one band migrating faster than the CP of PPV-M. This pattern was reproducible, and laboratory artifacts and mixed infections have been excluded [8]. Only a few PPV-Rec isolates have been shown to have a CP single-band profile in SDS-PAGE [9].

In this work, we compared the complete genome sequences of PPV-Rec isolates differing in their CP electrophoretic phenotypes and then used site-directed mutagenesis to demonstrate that a single amino acid mutation at position 66 of the CP can be responsible for a change in the behavior of the PPV-Rec CP in SDS-PAGE.

cDNA clone pIC-PPV [10] as well as the PPV isolates o6 [11], Š3 [12] and SK68 [13] were kindly provided by Prof. J. A. García (CSIC Madrid), Dr. M. Navrátil (PU Olomouc) and Prof. L. Palkovics (CU, Budapest), respectively. The other isolates used here were from the virus collection at IV SAS [3].

For PCR amplification of viral sequences, reverse transcription was performed using random hexamer primers and AMV reverse transcriptase (Promega) with total RNA isolated from virus-infected *Nicotiana benthamiana* plants by the RNeasy Plant Mini Kit (Qiagen). PCR of the (C-ter)NIb-(N-ter)CP genomic region (nt 7713-9061, numbering of the BOR-3 sequence, AY028309) was done using primers NSP4 for (TGGGACAAACTGCTTAGAGC) and NCuniR (CG CTTAACTCCTTCATACCAAG) and AccuTaq LA DNA polymerase (Sigma) under following conditions: 5 min

Z. W. Šubr (✉) · S. Nováková · A. Nagyová · M. Glasa
Institute of Virology, Slovak Academy of Sciences,
Dúbravská 9, 84505 Bratislava, Slovakia
e-mail: virusubr@savba.sk

M. Kamencayová · J. Nosek
Faculty of Natural Sciences, Commenius University,
Mlynská dolina, 84215 Bratislava, Slovakia

Published online: 28 April 2010

denaturation (95°C), 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 1.5 min, and 10 min final extension (72°C). FastDigest restriction enzymes *Sac*I and *BstX*I (Fermentas) were used to digest pIC-PPV and (C-ter)NIb-(N-ter)CP amplicons that had been purified using a Spinprep PCR Cleanup Kit (Novagen). DNA fragments were isolated from an agarose gel using the QIA Quick Gel Extraction Kit (Qiagen) and ligated. *E. coli* JM109 cells were used for cloning.

Viral genome sequences were obtained by the primer-walking method (Bitcet Bratislava) and analyzed using MEGA4.1 software [14] and the bioinformatic tools available at EMBL-EBI (http://www.ebi.ac.uk/) and CBS [15] (http://www.cbs.dtu.dk/services/).

For site-directed mutagenesis, the NSP4for/NCuniR-amplified PCR product of PPV isolate BULG was cloned in pGEM-T Easy (Promega). A single nucleotide exchange (italic and underlined in the primer sequences) was then introduced using primers GACCAGTTTCTCCAATTTC A*G*GGGCCACACCGC and GCGGTGTGGCCC*C*TGAA ATTGGAGAAACTGGTC and the QuickChange2 XL Kit (Stratagene) according to the manufacturer's instructions. The mutagenized fragment was then ligated into pIC-PPV using *Sac*I and *BstX*I restriction sites as described before. All chimeric PPV infectious cDNAs were verified by DNA sequencing.

Inoculation of plants with the various PPV cDNA clones was performed using the PDS-1000 He (Bio-Rad) biolistic apparatus following the manufacturer's instructions, and 0.5 µg of DNA was used for each bombardment of two potted plants (tungsten microcarriers M-10, shooting distance about 5 cm, helium pressure 1,100 psi). Analysis of crude plant sap was done 2 weeks later by immunoblotting with a polyclonal anti-PPV antibody [16].

Differences in electrophoretic mobility of the CPs of different PPV isolates cannot be explained solely by differences in the molecular weight of the CP. For example, with the exception of isolate Š-3, which has a significantly smaller CP due to a 28-aa deletion near its N terminus [12], the CPs of the analyzed isolates have calculated molecular weights of between 36.5 and 36.7 kDa but estimated molecular weights deduced from their electrophoretic mobility of between 38.7 and 40.7 kDa (Fig. 1c). Since PPV CP has been shown to be both glycosylated and phosphorylated [9, 17], one hypothesis is that the observed differences in electrophoretic mobility could reflect differences in posttranslational modifications of the CP from various isolates/strains of PPV.

The double-band CP pattern of PPV-Rec has been shown to be phosphatase-sensitive, suggesting the involvement of phosphorylation in this phenotype [18]. However, three PPV-Rec isolates of different geographic origin that show a single-band pattern are known from

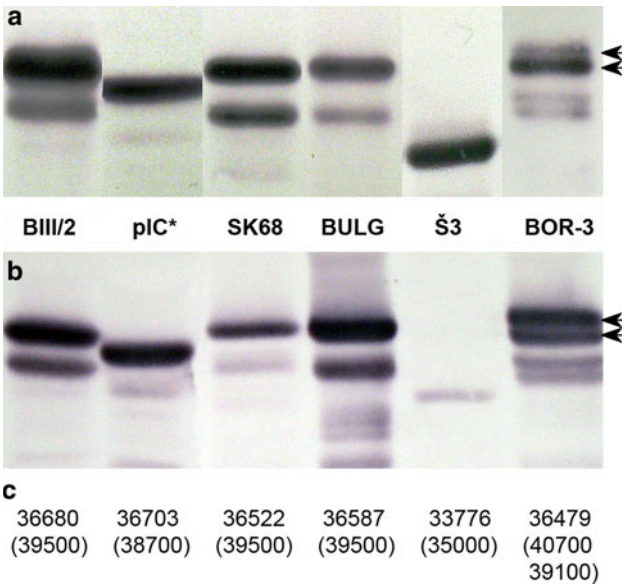

Fig. 1 Coat protein (CP) SDS-PAGE electrophoretic phenotypes of six PPV isolates. The isolate corresponding to the unmodified pIC-PPV is marked by an *asterisk*. *Full arrows* indicate the typical double-band of PPV-Rec. The *lower bands* in most samples correspond to CP degradation products. **a** Immunoblotting of the sap from plants infected by wild-type isolates. **b** Immunoblotting of the sap from plants infected by cDNA pIC-PPV-based chimeras with the (Cter)NIb-(Nter)CP part of the genome of the corresponding isolates exchanged. **c** Calculated and experimental molecular weights of the respective CPs. The values estimated by migration rates in SDS-PAGE are given in parentheses

Bulgaria (BULG, Fig. 1a), Serbia (o6) and Germany (Asl1) [3, 11].

The electrophoretic mobility of CP does not reflect the host species in which the virus is multiplied, including herbaceous and woody plants (data not shown), indicating that differences in phenotypes depend only on the viral genome. To test if non-structural PPV proteins influence the electrophoretic mobility of CP, we constructed chimeric cDNA clones bearing the (C-ter)NIb-(N-ter)CP genomic region from five PPV isolates exchanged with the corresponding region of the PPV-D infectious clone pIC-PPV. The isolates used represent three different PPV strains (PPV-D, PPV-M and PPV-Rec) and a range of electrophoretic phenotypes (Fig. 1a). The exchanged genome part did not influence symptom expression (data not shown), but the electrophoretic CP patterns of the constructed chimeras corresponded to those of the original isolates, as shown in Fig. 1b. This result demonstrates that the electrophoretic mobility of CP depends only on the N-terminal CP sequence and excludes an involvement of 5′-proximal non-structural genes in determining its electrophoretic mobility.

Two PPV-Rec isolates with atypical CP electrophoretic profiles (BULG and o6) were subjected to complete genome sequencing (GenBank GU461889 and GU474956).

When compared with other known complete PPV-Rec sequences (BOR-3, AY028309 and J4c, EU117116), a low degree of variability was detected, with identity levels of 98.07–99.17% for nucleotide sequences and 98.88–99.33% for deduced protein sequences. The CPs of isolates o6 and BOR-3 differ by five amino acids, while isolates BULG and BOR-3 differ by only two amino acids (Fig. 2), suggesting that the difference in CP electrophoretic behavior between these two isolates (Fig. 1a, b) is caused by mutations at positions 66 (Gly/Arg) and/or 135 (Gln/His). Further comparisons with CP sequences of Serbian PPV-Rec isolates having a CP double-band pattern (AY690609, AY690605) [19] excludes the involvement of the amino acid at position 135 in the phenotype change (Fig. 2).

To confirm the role of the mutation at position 66, site-directed mutagenesis was used to replace the Arg(66) in the BULG CP in a pIC-PPV chimera by a Gly residue. Six clones of the mutated chimeric construct pIC-BULG/Gly(66) were obtained and their sequence verified. All of them were infectious in *N. benthamiana* and, as shown in Fig. 3, the electrophoretic CP phenotype of the mutated virus was clearly double-band, contrasting with the single-band profile of the original BULG isolate or of the original, unmutated pIC-PPV-BULG chimera (Figs. 1a, b, 3). The difference in relative intensity of two bands observed is typical of the pattern observed with PPV-Rec isolates, with a stronger lower band (see, for example, the BOR-3 isolate in Fig. 1a). These results demonstrate that a single amino acid exchange in the viral CP markedly change the electrophoretic profile of CP. This result further reinforces the conclusion that the electrophoretic mobility of PPV CP does not solely reflect its molecular weight, since the difference between the exchanged amino acids is only 99 Da.

It is notable that the mutation at position 66 is not shared by the o6 isolate, which also shows a CP single-band phenotype. However, two other amino acids very close to this position are different in the o6 sequence (Fig. 2), supporting the hypothesis that the sequence (and possibly conformation) of this CP region contributes to the CP electrophoresis phenotype, possibly by modulating its post-translational modification(s).

Previous results indicated that the PPV CP is phosphorylated at several sites along the whole molecule, while its glycosylation sites were mapped outside of the conserved central core CP, probably in the N-terminal protruding region [9]. Pérez et al. [20] mapped two O-GlcNAc modification sites in this part of the PPV-D CP, namely Thr(19) and Thr(24). These sites are localized close to the motif Ser-Pro-Ile-Leu (CP aas 25-28), which is conserved only in PPV-D isolates but resembles the region of the PPV-Rec CP around the Gly(66)Arg mutation identified here: Ser-Pro-Ile-Ser-Gly/Arg (CP aas 62-66) (boxed in

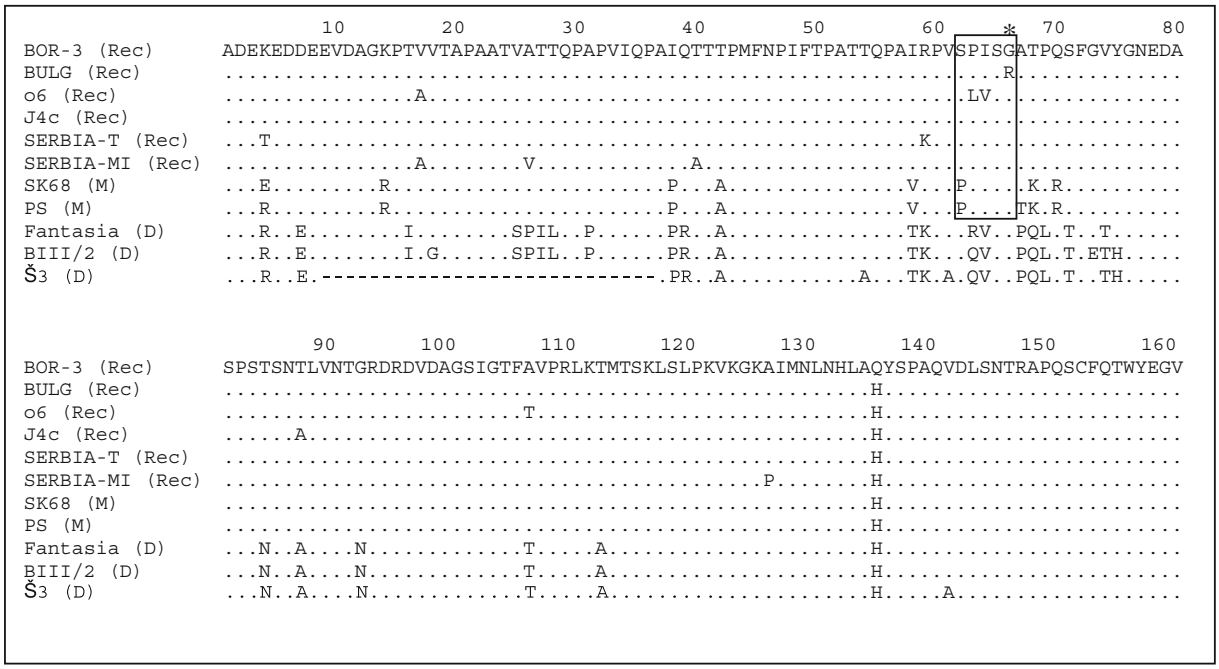

Fig. 2 Comparison of N-terminal CP amino acid sequences of selected PPV isolates. In addition to isolates analyzed in this work, representative isolates of the strains PPV-Rec, PPV-D and PPV-M are shown (AY028309 (BOR-3), EU117116 (J4C), AY690609 (SERBIA-T), AY690605 (SERBIA-MI), M92280 (SK68), AY912056 (Fantasia), GU461890 (BIII/2)). The BOR-3 sequence is shown at the *top*, amino acids of the other sequences identical to BOR-3 are represented by *dots*. The potential motif influencing the CP electrophoretic phenotype in PPV-Rec is *boxed*, and the target position for site-directed mutagenesis (66) is marked by an *asterisk*

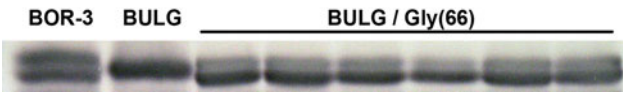

Fig. 3 Immunoblotting of PPV isolate BULG and of six clones of pIC-BULG with a mutated amino acid at position 66 (Gly instead of Arg). PPV isolate BOR-3 is shown in the first *line* for comparison

Fig. 2). Several modifiable amino acids are located in or close to this region, and indeed, in silico analysis (NetPhos, NetOGlyc) indicates that Ser(62) and Thr(68) are the best phosphorylation and *O*-glycosylation candidates in PPV-Rec CP (data not shown).

The sensitivity of the BOR-3 CP double-band pattern to in vitro dephosphorylation suggests the existence of two CP forms with different phosphorylation status [18]. The results presented here could indicate that the amino acid at position 66 may influence the accessibility of Ser(62) for a plant kinase, with Gly(66) in BOR-3 but not Arg(66) in BULG alllowing partial phosphorylation of Ser(62).

Posttranslational modifications of potyviral CPs have only recently been detected, and their biological function(s) remain unclear. Phosphorylation of the potato virus A CP has been shown to influence its RNA binding ability [21], while a PPV mutant unable to glycosylate CP progressed slowly in infected plants [22]. Both types of modification are common in eucaryotic cells and often occur close together or in competition. They have a high importance in cell signalling by changing protein activity [23]. Connections of PPV CP modifications with strain- or isolate-specific properties such as host or vector preference cannot be excluded. As the potyviral CP is known to be a multifunctional protein that participates in several processes during the virus life cycle [24], identifying possible regulatory roles for its modification(s) remains a challenge for further research.

Acknowledgments This work was supported by the grants 2/0027/09 from the Scientific Grant Agency of Ministry of Education and Slovak Academy of Sciences, APVV-51-0402-07 from Slovak Research and Development Agency, and partially by the European Union through the FP7 Small Collaborative Project KBBE-204429 (SHARCO acronym). The authors thank Prof. J.A. García, Prof. L. Palkovics and Dr. M. Navrátil for kindly providing the virus isolates and infectious cDNA. We are also grateful to Prof. J.A. García and Dr. T. Candresse for critical reading of the manuscript and for useful comments.

References

1. García JA, Cambra M (2007) Plum pox virus and sharka disease. Plant Viruses 1:69–79
2. Glasa M, Candresse T (2005) Partial sequence analysis of an atypical Turkish isolate provides further information on the evolutionary history of Plum pox virus (PPV). Virus Res 108:199–206
3. Glasa M, Palkovics L, Kominek P, Labonne G, Pittnerova S, Kudela O, Candresse T, Šubr Z (2004) Geographically and temporally distant natural recombinant isolates of Plum pox virus (PPV) are genetically very similar and form a unique PPV subgroup. J Gen Virol 85:2671–2681
4. Serce CU, Candresse T, Svanella-Dumas L, Krizbai L, Gazela M, Caglayan K (2009) Further characterization of a new recombinant group of Plum pox virus isolates, PPV-T, found in orchards in the Ankara province of Turkey. Virus Res 142:121–126
5. Šubr Z, Glasa M (2008) Plum pox virus variability detected by the advanced analytical methods. Acta Virol 52:75–90
6. Bousalem M, Candresse T, Quiot-Douine L, Quiot JB (1994) Comparison of three methods for assessing plum pox virus variability: further evidence for the existence of two major groups of isolates. J Phytopathol 142:163–172
7. López-Moya JJ, Canto T, López-Abella D, Díaz-Ruíz JR (1994) Differentiation of Mediterranean plum pox virus isolates by coat protein analysis. Plant Pathol 43:164–171
8. Šubr Z, Glasa M (1999) Plum pox virus capsid protein mobility in SDS-polyacrylamide gel electrophoresis. Acta Virol 43:259–262
9. Kollerová E, Glasa M, Šubr ZW (2008) Western blotting analysis of the Plum pox virus capsid protein. J Plant Pathol 90:S1.19–S1.22
10. López-Moya JJ, García JA (2000) Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. Virus Res 68:99–107
11. Cervera MT, Riechmann JL, Martin MT, Garcia JA (1993) 3′ terminal sequence of the plum pox virus PS and o6 isolates: evidence for RNA recombination within the potyvirus group. J Gen Virol 74:329–334
12. Navrátil M, Šimonová V, Fialová V, Válová P (1998) Molecular variability of Czech plum pox virus isolates. Acta Virol 42:254–256
13. Tóbiás I, Palkovics L, Tzekova L, Balázs E (2001) Replacement of the coat protein gene of plum pox potyvirus with that of zucchini yellow mosaic potyvirus: characterization of the hybrid potyvirus. Virus Res 76:9–16
14. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599
15. Julenius K, Mølgaard A, Gupta R, Brunak S (2005) Prediction, conservation analysis, and structural characterization of mammalian mucin-type *O*-glycosylation sites. Glycobiology 15:153–164
16. Šubr Z, Matisová J (1999) Preparation of diagnostic monoclonal antibodies against two potyviruses. Acta Virol 43:255–257
17. Fernández-Fernández MR, Camafeita E, Bonay P, Méndez E, Albar JP, García JA (2002) The capsid protein of a plant single-stranded RNA virus is modified by *O*-linked *N*-acetylglucosamine. J Biol Chem 277:135–140
18. Šubr Z, Ryšlavá H, Kollerová E (2007) Electrophoretic mobility of the capsid protein of the plum pox virus strain PPV-Rec indicates its partial phosphorylation. Acta Virol 51:135–138
19. Glasa M, Paunovic S, Jevremovic D, Myrta A, Pittnerová S, Candresse T (2005) Analysis of recombinant Plum pox virus (PPV) isolates from Serbia confirms genetic homogeneity and supports a regional origin for the PPV-Rec subgroup. Arch Virol 150:2051–2060
20. Pérez JJ, Juárez S, Chen D, Scott CL, Hartweck LM, Olszewski NE, García JA (2006) Mapping of two *O*-GlcNAc modification sites in the capsid protein of the potyvirus Plum pox virus. FEBS Lett 580:5822–5828
21. Ivanov KI, Puustinen P, Gabrenaite R, Vihinen H, Rönnstrand L, Valmu L, Kalkkinen N, Mäkinen K (2003) Phosphorylation of the potyvirus capsid protein by protein kinase CK2 and its relevance for virus infection. Plant Cell 15:2124–2139
22. Chen D, Juárez S, Hartweck L, Alamillo JM, Simón-Mateo C, Pérez JJ, Fernández-Fernández MR, Olszewski NE, García JA

(2005) Identification of secret agent as the *O*-GlcNAc transferase that participates in Plum pox virus infection. J Virol 79:9381–9387

23. Slawson C, Hart GW (2003) Dynamic interplay between *O*-GlcNAc and *O*-phosphate: the sweet side of protein regulation. Curr Opin Struct Biol 13:631–636
24. Callaway A, Giesman-Cookmeyer D, Gillock ET, Sit TL, Lommel SA (2001) The multifunctional capsid proteins of plant RNA viruses. Annu Rev Phytopathol 39:419–460