# ELECTROPHORETIC MOBILITY OF THE CAPSID PROTEIN OF THE PLUM POX VIRUS STRAIN PPV-REC INDICATES ITS PARTIAL PHOSPHORYLATION

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**Summary. –** A double-band SDS-PAGE profile was found reproducible for capsid protein (CP) of Plum pox virus (PPV) isolates belonging to the strain PPV-Rec. The double-band was also present in the virus population multiplied in various plants. A single-lesion passage in a hypersensitive host *Chenopodium foetidum* showed that its presence was not a result of a mixed infection. We found that the two electrophoretic forms of CP shared identical N-terminus. Therefore, they did not originate from an alternative proteolytic processing, but were different in their posttranslational modification. The slower band of CP could be converted to the faster one by the phosphatase treatment. We assumed that CP protein was present in both phosphorylated and dephosphorylated forms in the infected plants.

**Key words:** electrophoretic mobility; phosphorylation; potyvirus

PPV is an aphid-transmissible potyvirus causing "sharka" disease of stone fruit trees. In filamentous viral particles the genomic (+)ssRNA is encapsidated by approximately 2,000 copies of a single type of CP (Riechman *et al*., 1992). Currently, 6 strains of PPV are recognized: three of them are of lower economic importance, because of their restricted geographical distribution (strains PPV-EA, PPV-W) or narrow host specificity (PPV-C). On the other hand, the remaining three strains PPV-D, PPV-M and PPV-Rec seriously threaten the plum, apricot and peach orchards in European and Mediterranean region. The strains PPV-D and PPV-M are recognized as serotypes (Kerlan and Dunez, 1979). The strain PPV-Rec cannot be differentiated from PPV-M in serological tests, since its genome is based on the homologous recombination between PPV-D and PPV-M genomes upstream the CP gene (Glasa *et al*., 2004).

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**Abbreviations:** CP = capsid protein; PPV = Plum pox virus; PVDF = polyvinylidene fluorid

A different CP mobility of PPV-M and PPV-D was described in SDS-PAGE (Adamolle, 1993). CP of some PPV isolates detected by immunoblotting with polyclonal and monoclonal antibodies migrated in the form of a double band. This phenomenon has been attributed to the partial CP degradation during infection of plants or virus purification eventually to mixed infections (Komínek *et al*., 1997; Navrátil *et al*., 1998; Šubr and Glasa, 1999). We showed in this study that the presence of a double CP band was specific for the strain PPV-Rec, and that this phenomenon was produced by a posttranslational modification.

The isolates BOR-3 (strain PPV-Rec), BULG (strain PPV-Rec), KR-4 (strain PPV-M) and Dideron (strain PPV-D) (Glasa *et al*., 2004; Kerlan and Dunez, 1979) were propagated mechanically in *Nicotiana benthamiana* and purified according to Lain *et al*. (1988). *Ch. foetidum* plants were infected and single lesions were cut off the leaves and reinoculated to *N. benthamiana*. The samples of crude plant sap or purified virus were analyzed by SDS-PAGE in 10% gels (Laemmli, 1970) and by immunoblotting (Towbin *et al*., 1979). The gels were stained by Coomassie Brilliant Blue (CBB) or by silver nitrate (Marcinka *et al*., 1992) and the blots were immunostained with polyclonal anti-PPV antibody (Šubr and Mati-

sová, 1999). CP bands of the isolate BOR-3 separated by SDS-PAGE were transferred to PVDF membrane, stained by CBB and subjected to N-terminal amino acid sequencing by LF 3600D Protein Sequencer (Beckman Instruments). For the dephosphorylation of CP, 1 µl of the purified PPV ($A_{260}$ 5.2, $A_{280}$ 4.1) was incubated for 2 hrs at 37°C with 6 U of calf intestinal phosphatase (Fermentas) and analyzed by immunoblotting.

Initially, we observed the double-band migration profile for the isolate BOR-3 (Šubr and Glasa, 1999). The double-band was detected reproducibly in crude sap samples from various woody and herbaceous host plants including *Prunus persica, P. domestica, N. benthamiana, N. clevelandii* and *Pisum sativum*, as well as in purified virus. To exclude the possibility of mixed infection we inoculated BOR-3 on a local lesion host *Ch. foetidum*. Subisolates of BOR-3 obtained from individual necrotic lesions were analyzed by immunoblotting. Each subisolate showed identical double-band pattern as the original isolate BOR-3 indicating, that this phenomenon was not produced by a mixed infection (Fig. 1). Evidently, the presence of double-band pattern was characteristic for the viral isolate itself, because it was highly reproducible and independent from the host plant or purification method used.

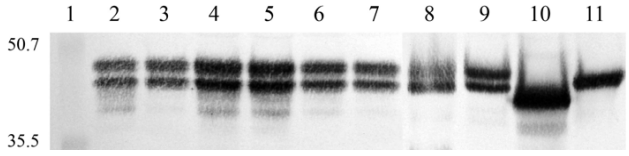


**Fig. 1**

**Immunoblot analysis of BOR-3 subisolates obtained from single lesions in *Ch. foetidum* (lanes 2–8)**

Purified isolates BOR-3 (lane 9), Dideron (lane 10) and BULG (lane 11) used as controls. MW markers (lane 1)

The isolate BOR-3 is the type member of recently recognized PPV-Rec strain originating from an ancestral homologous recombinantion between strains PPV-M and PPV-D (Glasa *et al*., 2004). Immunoblotting of 85 PPV isolates (including strains PPV-M, PPV-D and PPV-Rec) combined with restriction fragments length polymorphism analyses and partial genome sequencing enabled us to confirm that isolates with the double-band pattern belonged exclusively to the strain PPV-Rec (Glasa *et al*., 2004 and unpublished results). Conversely, nearly all of 39 PPV-Rec isolates confirmed by genome sequencing shared double-band CP pattern with the exception of CP of two isolates migrating as a single band with the same electrophoretic mobility as CP of PPV-M. The electrophoretic pattern of CP of typical (BOR-3) and non-typical (BULG) PPV-Rec isolates are compared to the strains PPV-M and PPV-D (Fig. 2).

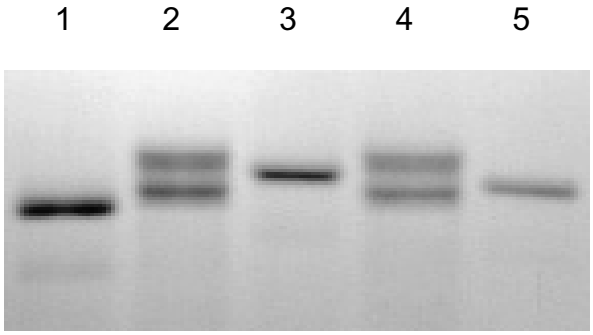


**Fig. 2**

**SDS-PAGE of silver-stained CP in various PPV isolates**

Lane 1: Dideron (PPV-D), lanes 2, 4: BOR-3 (PPV-Rec), lane 3: KR-4 (PPV-M), lane 5: BULG (PPV-Rec).

Generally, there are two possibilities to explain different mobility of proteins in SDS-PAGE – different length or different posttranslational modification of the protein. Potyviral polypeptides are released from a virus-coded polyprotein by the action of 3 viral proteases. The CP polypeptide is split off the rest of polyprotein by the NIa protease (Riechmann *et al*., 1992). Although the aa identity of NIa proteases in strains PPV-M and PPV-D is relatively high (96.5%), most of their aa differences are accumulated in the C-terminal region close to the bound substrate, what may contribute to the recognition of the target aa sequence (Mestre *et al*., 2003). While the NIa gene is localized upstream the recombination point in PPV-Rec, the CP gene including the whole sequence needed for its cleavage (recognized by the NIa protease) lies downstream this point. As a result, in the strain PPV-Rec the protease is of "D-type" and its cleavage site of "M-type", which may theoretically lead to an incorrect proteolysis and appearance of CP populations with different N-termini and consequently with different polypeptide length. To test this hypothesis we separated the two CP bands of PPV-Rec (isolate BOR-3) by SDS-PAGE. Each protein band was analyzed by direct N-terminal amino acid sequencing. For the each protein band we determined first 10 aa from N-terminus and found that both polypeptides started with identical aa sequence predicted by nucleotide sequence of BOR-3 genomic RNA (Glasa and Šubr, 2005; GenBank Acc. No. AY028309). Because the 3'-ends of CP genes in different PPV strains are highly conserved, there is no reason to presume an alternative translation finishing (e.g. by a read-through mechanism) in recombinant isolates. Thus, the presence of double CP band in PPV-Rec isolates may be caused by a posttranslational modification of a part of the CP population.

There are limited data available about non-peptide compounds in capsids of plant (+)ssRNA viruses. Recently, the glycosylation and phosphorylation of CP was demonstrated for PPV isolates Rankovic (PPV-D) and PS (PPV-M) (Fernández-Fernández *et al*., 2002). We analyzed the electrophoretic mobility of CP of isolates KR-4 (PPV-M), Dideron (PPV-D), BULG (PPV-Rec with single CP band pattern) and PPV BOR-3 (PPV-Rec with double CP band pattern) after dephosphorylation. The CP of isolates KR-4, Dideron, and BULG showed no shift in their electrophoretic mobilities after phosphatase treatment. In contrast, the isolate PPV BOR-3 showed almost complete disappearance of slower migrating band after phosphatase treatment (Fig. 3). Thus, the double CP band pattern of PPV-Rec seemed to originate from the phosphorylation of about half of CP population.

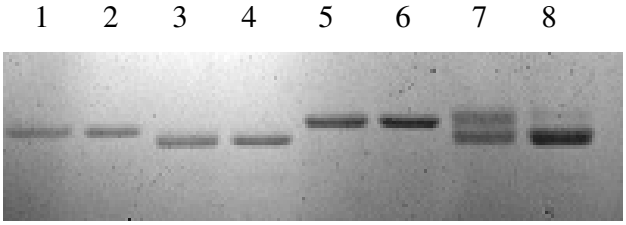


**Fig. 3**

**SDS-PAGE of CBB-stained CP in purified virus isolates BULG, Dideron, KR-4 and BOR-3, respectively, before (1, 3, 5, 7) and after (2, 4, 6, 8) treatment with phosphatase**

"Core CP" obtained by mild virion trypsinolysis of PPV BOR-3 migrated as a single band (Šubr and Glasa, 1999) indicating, that the modified region was localized near one or both of the CP termini. The N-terminal region of CP is known as the most variable region among strains and isolates of the genus *Potyvirus* (Shukla and Ward, 1988). On the other hand, the C termini of CP of different PPV isolates are highly conservative. We compared the N-terminal sequences of CP in several PPV-Rec and PPV-M isolates accessible in Gen Bank. Their accession numbers are: 89-006 (AY324842), VAR-2 (AY324837), Nectagrand (AY324839), Pd31 (AJ566345), Pd4 (AJ566344), Slivoň (AY324843), BULG (AY324846), Bt-H2 (AJ566346) and BOR-3 (AY028309). Although the CP of PPV-Rec isolates was principally of the "M-type", we found six amino acids in N-terminal region conserved in all sequenced isolates that were different from conventional PPV-M isolates (Fig. 4).

Two of these exchanged aa T2852 and S2872 potentially may be phosphorylated. Results obtained by phosphorylation prediction at NetPhos 2.0 Server (Blom *et al*., 1999) showed that the T2852A and I2848P substitutions enhanced the probability of T2852 phosphorylation. The S2872 showed

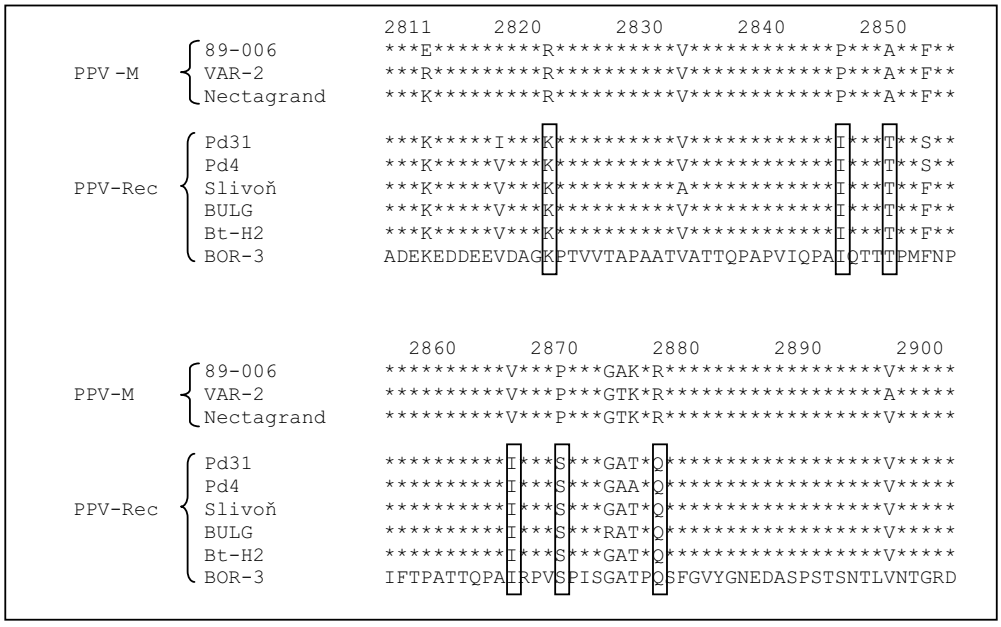


**Fig. 4**

**Comparison of aa sequences of N-terminal region of CP from several PPV-M and PPV-Rec isolates**

The numbers express the aa positions in PPV polyprotein, aa conserved in PPV-Rec and different from PPV-M are boxed.

high score in the phosphorylation prediction itself. The "single-band" isolate BULG shared all these PPV-Rec typical aa, therefore we supposed that some additional viral factors must be responsible for the CP modification in typical PPV-Rec isolates. So far, no kinase/phosphatase activity associated with any of the potyviral gene products has been found. Thus, the difference in CP phosphorylation may be considered as a consequence of the host enzymatic activity. However, this enzymatic activity did not depend on the host plant species, because identical double CP bands were observed in infected systemic and hypersensitive as well as woody and herbaceous plants. Thus, we supposed that CP was using the host enzymatic machinery probably controlled by the virus for its phosphorylation.

The problem remained why only a part of the CP population was phosphorylated. Although the phosphorylation of potyviral CP was detected, it has never been associated with the presence of two distinct electrophoretic forms corresponding to phosphorylated and dephosphorylated form of CP (Ivanov *et al*., 2001; Fernández-Fernández *et al*., 2002). This is the first report describing identification of phosphorylated and dephosphorylated form of potyviral CP in infected plants. We suppose that both forms of CP may be set to different roles in infected cells. Potyviral CP is multifunctional protein playing role in viral genome encapsidation, in vector transmission, cell-to-cell and long distance movement (Revers *et al*., 1999). Phosphorylation is one of most important regulation mechanisms in eukaryotic cells. It is possible that CP phosphorylation contributes to the high capability of PPV-Rec isolates to overcome the host defense mechanism, what advances to a broad spread of these viruses in central and southern Europe.

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