

## WESTERN BLOTTING ANALYSIS OF THE *PLUM POX VIRUS* CAPSID PROTEIN

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### SUMMARY

Immunoblotting using a polyclonal antiserum to *Plum pox virus* (PPV) was used to detect PPV capsid protein (CP) in different parts of mechanically infected *Nicotiana benthamiana* plants from 1 to 21 days post inoculation (dpi). The velocity of viral spread in infected plants depended only slightly on the inoculum concentration. Roots and tops of the plants became infected 5-7 dpi. Systemic infection of non-inoculated leaves in the middle part of the plants was detected later (7-10 dpi), but infective viral RNA proved to be there 1 dpi. Comparing several isolates of PPV strains M, D and Rec, the fastest infection movement was detected for PPV-Rec, the slowest for PPV-D. Differences in the CP electrophoretic mobility among the strains M, D and Rec did not correspond to their amino acid sequences. Post-translational CP glycosylation and phosphorylation was detected in six PPV isolates. We presume the non-amino acid compounds linked to the N termini are responsible for the electrophoretic pattern differences among the studied isolates.

*Key words:* PPV strains, systemic movement, glycosylation, phosphorylation, coat protein.

### INTRODUCTION

Six strains of *Plum pox virus* (PPV) have been recognized according to genome sequence, immunochemical properties, host specificity and geographic distribution, i.e. PPV-M, PPV-D, PPV-EA, PPV-C, PPV-Rec and PPV-W (Kerlan and Dunez, 1979; Wetzell *et al.*, 1991; Nemchinov *et al.*, 1998; Glasa *et al.*, 2004; James and Varga, 2005). PPV-D, PPV-M and PPV-Rec are widely spread in Europe, especially in the central and south-eastern parts of the continent. PPV-Rec has evolved from a homologous recombination event between PPV-D and PPV-M in the viral replicase gene (Glasa *et al.*,

2001). As a consequence, PPV-Rec is hardly serologically distinguishable from PPV-M, although the majority of its genome is highly homologous to PPV-D.

A method for the rapid differentiation between the three major PPV strains is sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of their capsid proteins (CP), which differ in their mobility in gel (Adamolle, 1993; Šubr and Glasa, 1999). Posttranslational modifications of PPV CP have been recently identified (Fernández-Fernández *et al.*, 2002).

*Nicotiana benthamiana* is an experimental host for many plant viruses, including PPV, which infects it systemically reaching high titers. In comparison to woody hosts, this condition is especially useful for PPV multiplication and purification. Moreover, certain natural hosts show strain-specific sensitivity to this virus (e.g. PPV-D is less virulent in peaches, PPV-Rec is commonly found in plums, rarely in apricots and not in peaches, while PPV-M infects peaches readily) thus, systemic herbaceous plant species can be used for comparing virus isolates independently of their host specificity or preference.

We have already tested the relative infectivity of PPV isolates in mixed infections in *N. benthamiana* (Šubr *et al.*, 2004) and have now investigated six isolates of its three main strains for CP glycosylation and phosphorylation, comparing the relative systemic movement rates in *N. benthamiana*.

### MATERIALS AND METHODS

**Virus isolates.** PPV-Rec isolates BOR-3 and BULG, PPV-M isolates Kr-4 and SK-68, and PPV-D isolates Dideron and BIII/2 (Kerlan and Dunez, 1979; Palkovics *et al.*, 1993; Glasa *et al.*, 2004) were propagated in *N. benthamiana* and purified according to Laín *et al.* (1988). For infection kinetic studies, one leaf was mechanically inoculated and samples were collected at one-day interval from different plant parts including roots, inoculated and non-inoculated leaves (the one above the inoculated leaf) and growing tip.

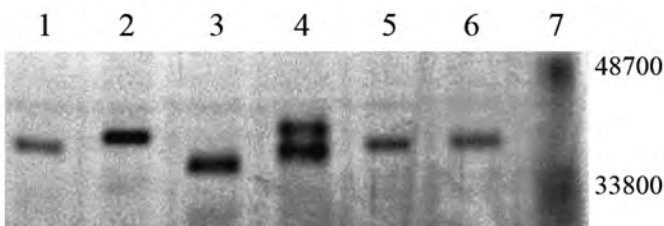
**Electrophoresis and Western blotting.** Samples were homogenized in phosphate-buffered saline solution (PBS, pH 7.0) 1:3 (w:v), centrifuged for 5 min at 16000g and the supernatant was mixed 1:1 with Laemmli sample buffer. Discontinuous SDS-PAGE in 10 % gels (Laemmli, 1970) was followed by silver staining (Marcinka *et al.*, 1992) or Western blotting (Nováková *et al.*, 2006) and immunostaining using a rabbit polyclonal anti-PPV antiserum (Šubr and Matisová, 1999). Glycoproteins were detected by the GelCode Glycoprotein Staining Kit (Pierce, USA) and phosphoproteins by the OMNI-PHOS Phosphorylation Assay Kit (Chemicon, USA), according to the manufacturers' instructions.

**CP digestion.** Core CP was prepared by treatment of purified PPV with trypsin (1 µg/ml) at room temperature for 1 h. The virus was then subjected to ultracentrifugation (110,000g for 2 h) and sedimented core CP-containing particles were resuspended in PPV storage buffer (Lain *et al.*, 1988).

**RT-PCR.** Viral RNA from infected plants was amplified by RT-PCR of total RNA isolated by the RNeasy kit (Qiagen, USA) using primers 5'-GTACGATATCTC-CGTTCGGT-3' and 5'-TGGAGTTGATCCAAAG-GTGC-3' which amplify a fragment of 1033 bp of the P3-6K1-CI genome region. Reverse transcription was done as described previously (Glasa *et al.*, 2002). PCR conditions were: 35 cycles at 95°C for 30 sec, 58°C for 40 sec and 72°C for 1.5 min.

## RESULTS AND DISCUSSION

**CP modifications.** Different CP mobility has been previously reported for PPV-M and PPV-D (Adamolle, 1993; Bousalem *et al.*, 1994) and CP of PPV-Rec was shown to migrate in a double-band form in SDS-PAGE (Šubr and Glasa, 1999; Šubr *et al.*, 2007). Although these electrophoretic patterns were shared by most of PPV isolates tested in our laboratory, few isolates (in-

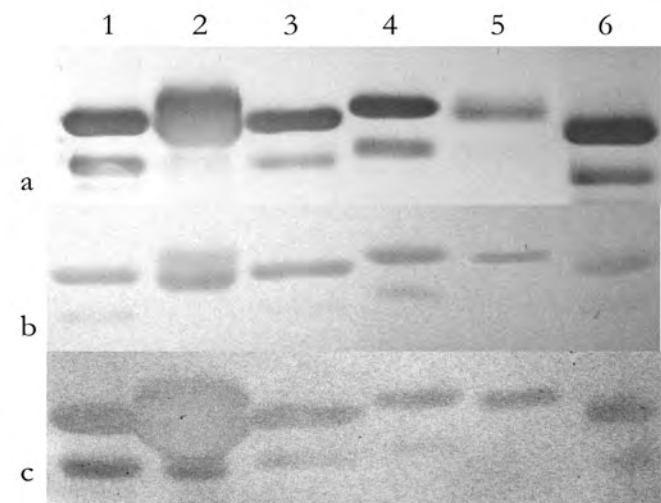


**Fig. 1.** SDS-PAGE of the capsid protein of *Plum pox virus*, silver-stained gel. Isolates BULG (lane 1), Kr-4 (lane 2), SK68 (lane 3), BOR-3 (lane 4), Dideron (lane 5) and BIII/2 (lane 6), Molecular weight marker (lane 7).

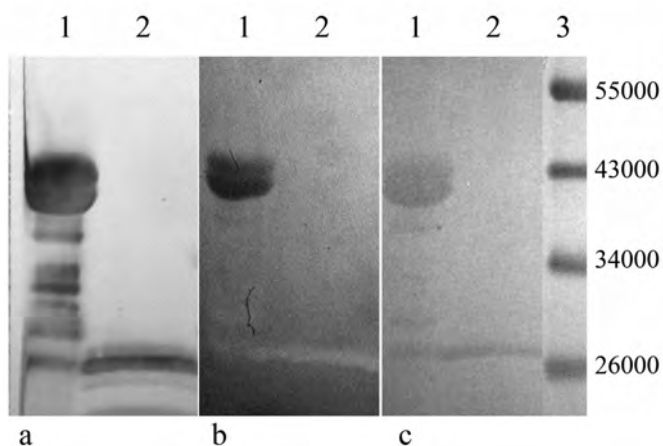
cluding BULG, BIII/2, Kr-4) showed atypical CP mobility (Fig. 1). The lack of SDS binding by carbohydrates leads to a lower charge-to-mass ratios for SDS-glycoprotein complexes and to an over-estimation of their molecular weights (MW) by SDS-PAGE (Werner *et al.*, 1993).

According to single PAGE in 10% gels the mol. wt of PPV CP ranged from 38.7 to 40.7 kDa. Ferguson plots (Ferguson, 1964), based on 7-15% gels, indicated that CP mobilities reflected their modifications by non-amino acid compounds (data not shown) and comparison of theoretical mol. wt calculated from the known CP amino acid sequences confirmed this finding (Table 1). At least two PPV isolates have been shown to bear glycosidic and phosphate residues on their CP (Fernández-Fernández *et al.*, 2002) and phosphorylation has been shown to play a role also in the PPV-Rec double-band pattern (Šubr *et al.*, 2007). We used commercial kits for glycoprotein and phosphoprotein staining to confirm these modifications in the six PPV isolates. All of them were positive in both tests, including both bands of isolate BOR-3 (Fig. 2). Separate reactions for phospho-serine, phospho-threonine and phospho-tyrosine were all positive (data not shown).

Mild trypsinolysis of potyviral virions causes the digestion of CP termini, leaving a core CP with high homology, while most sequence differences (both at the strain and virus level) are localized in the N termini of CP subunits (Shukla *et al.*, 1988). In our case, the core CP was not glycosylated but it still gave a positive reaction with phosphoprotein staining (Fig. 3). As the core CP of different isolates migrates identically in SDS-PAGE (Šubr and Glasa, 1999), we assume that the in-



**Fig. 2.** Analysis of the capsid protein of *Plum pox virus* by different techniques. Immunostaining with anti-PPV antibody (row a), glycoprotein (row b) and phosphoprotein (row c) staining. Isolates BULG (lane 1), BOR-3 (lane 2), SK68 (lane 3), Kr-4 (lane 4), BIII (lane 5) and Dideron (lane 6).



**Fig. 3.** Analysis of the capsid protein (lanes 1) and core CP (lanes 2) of isolate BOR-3 by different techniques. Immunostaining with anti-PPV antiserum (panel a), glycoprotein (panel b) and phosphoprotein (panel c) staining. Molecular weight marker (lane 3).

tact CP mobility differences are mainly caused by the glycosylation at their N termini, which is in agreement with the analyses of digested PPV CP by mass spectrometry (Fernández-Fernández *et al.*, 2002).

**Systemic movement in *N. benthamiana*.** Kinetic studies of systemic PPV diffusion in *N. benthamiana* should compare the rate of spreading of different viral isolates and detect CP modifications *in vivo*. We could not detect unmodified CP forms in any part of the infected plants, for only the ordinary electrophoretic CP form of each isolate was found. Although RT-PCR detected viral RNA in non-inoculated leaves as soon as 1 dpi (and its infectivity was proven with a reinoculation experiment), the CP could be found later in the same tissues by immunoblotting (Table 2). The difference in

**Table 1.** Calculated and apparent molecular weight (MW) of the capsid protein of *Plum pox virus* isolates.

Virus strain	Virus isolate	Mol. wt (kDa)	
		Sequence	PAGE
Rec	BOR-3	36.48	40,7
			39,1
Rec	BULG	36.57	39,1
M	SK68	36.52	39,2
M	Kr-4	Nd <sup>(a)</sup>	39,9
D	Dideron	36.65	38,7
D	BIII/2	Nd	39,7

<sup>(a)</sup>not determined

**Table 2.** Systemic spread of *Plum pox virus* isolates in *Nicotiana benthamiana*. First detection (dpi) of viral capsid protein by immunoblotting in particular plant parts is recorded.

Virus strain	Virus isolate	Roots	NI <sup>(a)</sup> leaves	Top
Rec	BOR-3	5	7	5
Rec	BULG	5	8	5
M	SK68	5	10	5
M	Kr-4	5	10	4
D	Dideron	7	10	8
D	BIII/2	7	10	7

<sup>(a)</sup>NI = Non-inoculated

the rate of systemic spread of different strains was not significant. However, it was related to the strain affiliation of particular isolate, independently of the CP mobility pattern. Generally, PPV-D isolates were the slowest to become established in the plant and PPV-Rec the fastest. In strain-mixed infections, PPV-D isolates were more competitive than PPV-M in *N. benthamiana* (Šubr *et al.*, 2004). Therefore, it is possible that the relative virulence of particular isolates depends on other PPV-host interactions than the rate of systemic movement.

The spatio-temporal spread of PPV in the plant was similar for all tested isolates. Generally, the virus was first detected in both terminal tips (roots and apical tissues) and slightly later in non-inoculated leaves of the middle parts of the plant, suggesting that the primary virus systemic spread is very fast, following the source-to-sink flow of assimilates. Further virus replication is more massive within plant parts with intensive cellular metabolism (fast-dividing growing tip tissues). In infected woody hosts the situation is similar (Bodin-Ferri *et al.*, 2002). Although PPV distribution in the plant is much more uneven, the virus seems to multiply best in young growing sprouts. Although its presence in tree roots has not been studied extensively, according to the herbaceous model, it may play an important role in the infection persistence or intra-virus variability (Jridi *et al.*, 2006).

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