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# Tracking the potyviral P1 protein in *Nicotiana benthamiana* plants during plum pox virus infection

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**Summary. –** The P1 protein is derived from the N terminus of potyvirus-coded polyprotein. In addition to the proteolytic activity essential for its maturation, it probably participates in suppression of host defense and/or in virus replication. Clear validation of the P1 *in vivo* function(s), however, is not yet available. We applied an infectious cDNA clone of plum pox virus (PPV), where the P1 was N-fused with a hexahistidine tag, to trace this protein in *Nicotiana benthamiana* plants during the PPV infection. Immunoblot analysis with the anti-his antibody showed a diffuse band corresponding to the molecular weight about 70–80 kDa (about twice larger than expected) in the root samples from early stage of infection. This signal culminated on the sixth day post inoculation, later it rapidly disappeared. Sample denaturation by boiling in SDS before centrifugal clarification was essential, indicating strong affinity of P1-his to some plant compound sedimenting with the tissue and cell debris.

**Keywords:** potyvirus; his-tag fusion; immunoblotting

Genome expression of the plum pox virus (PPV, the genus *Potyvirus*) is based on the „polyprotein strategy". Particular polyprotein-derived polypeptides are multifunctial and participate in various processes including viral RNA replication, polyprotein processing, inhibition of posttranscriptional gene silencing, cell-to-cell and systemic movement, encapsidation and assistance at vector-based transmission (Šubr and Glasa, 2013). The N-proximal P1 protein is believed to be involved in suppression of host defense (Valli *et al*., 2006), in virus replication (Pasin *et al*., 2014) or stimulation of viral proteosynthesis (Martínez and Darós, 2014), however, its detailed function and mechanism of action during infection cycle remain unknown. Here we tried to trace the viral P1 in the systemic host *Nicotiana benthamiana* during an experimental PPV infection.

The infectious cDNA clone pIC-PPV-Rec (Predajňa *et al*., 2012) was modified by inserting the sequence coding for six histidine residues between the fourth and fifth amino acid of the P1 protein. This was performed using specific primers in two separate PCR resulting in overlapping fragments, which were then combined by another amplification step (Table 1). Final product was sequence-verified and replaced for the homologous region in the cDNA clone by *Pvu*I-*Rsr*II restriction/ligation to gain the construct pIC-PPV-Rec-P1His.

*N. benthamiana* plants were transfected biolistically using a previously developed air-gun system (Predajňa *et al*., 2010). Three weeks later, PPV was proved in the plants by immunoblotting and the presence of modified virus was verified by PCR and sequencing. The virus was further passaged by mechanical inoculation. Samples from various plant parts (inoculated and non-inoculated leaves, stems, apical parts and roots) were collected at various time-points after infection and analysed by immunoblotting with anti-histidine antibodies (Sigma). The tissues were grinded by mortar and pestle in PBS pH 7.4 (1:2, w:v) and subsequent denaturation step (5 min boiling in the SDS-PAGE sample buffer) was performed either before or after centrifugal removal of the cell and tissue debris (5 min at 16,000 x g).

The PPV with his tag-fused P1 was able to replicate in *N. benthamiana* and it remained stable during several mechanical passages of the virus. Ten days post inoculation (dpi), the viral capsid protein was clearly detectable

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**Abbreviations:** PPV = plum pox virus

**Table 1. Primers and PCR conditions used for construction of pIC-PPV-Rec-P1His**

| Primer (position)[1] | Sequence[2] | Template | PCR conditions[3] | Amplimer (length) |
|---|---|---|---|---|
| VekFor (12869–12889) | ATTAATGCAGCTGGCTTATCG | pIC-PPV-Rec | $T_a$ 56°C $t_e$ 2 min | 1 (1509 bp) |
| 6hisP1rev (137–161) | TACgtggtggtggtggtggtgAATGGTTG ACATCTTGACTTGC | | | |
| 6hisP1for (156–179) | ATTcaccaccaccaccaccacGTATTTGGCTCA TTCACTTGC | pIC-PPV-Rec | $T_a$ 56°C $t_e$ 40 s | 2 (453 bp) |
| BorRArev (569–590) | AGGTTTCTCAATAATATGAGGG | | | |
| VekFor | as above | amplimer 1 + amplimer 2 | $T_a$ 56°C $t_e$ 2.5 min | 3 (1920 bp) |
| BorRArev | as above | | | |

[1]Position numbered according to pIC-PPV-Rec (Predajňa *et al*., 2012); [2]inserted linker coding for hexahistidine tag is represented by lower case letters; [3]$T_a$ = annealing temperature, $t_e$ = elongation time.

in all inspected plant parts independently on the sample preparation method. Anti-his antibody, however, gave only weak diffuse signal in immunoblot (prolonged incubation with the substrate was needed, resulting in a relatively high background), which became much stronger in root samples when the denaturation step preceded the centrifugal clarification (Fig. 1a). Similar behavior has been recently described for *in planta* expressed PB1-F2 protein of influenza virus, known to create insoluble amyloid fibres (Kamencayová *et al*., 2014). Therefore we presume P1-his low solubility either as its intrinsic feature, or as a result of its affinity to some plant compound sedimenting with the debris.

The observed diffuse band from the root samples corresponded to molecular mass between 70–80 kDa, which was about twice higher than expected (≈36 kDa). This might be due to any posttranslational modifications (including covalent dimerisation). An atypical release from the polyprotein cannot be excluded, too, although the P1 protease domain is located on its C terminus (Valli *et al*., 2007) and is unlikely to be affected by the N-proximal fusion partner. At least partial purification will be needed for further characterisation of this polypeptide. Interestingly, no (or very weak) reaction with anti-histidine antibody was observed in samples from other plant tissues, while the root sample was clearly positive during the early infection stage. It may be caused by extremely fast degradation of P1 in the green parts *in vivo* or during sample preparation. Even in root samples, the turnover of P1 was very fast. The signal strength culminated at 6 dpi, later it rapidly disappeared (Fig. 1b). Twin-Strep tag-fused P1 of Tobacco etch potyvirus has been proved in systemically infected leaves of *N. benthamiana* and its appearance during early stage of infection correlated very well with our results (Martínez and Darós, 2014).

P1 is the most divergent potyviral protein, therefore its role in specific interactions with particular host factors has

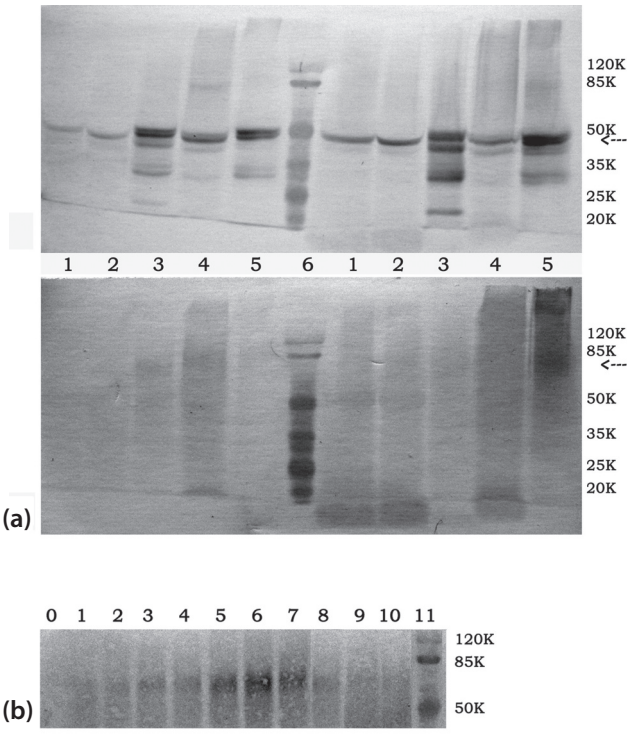


**Fig. 1**

**Detection of PPV P1-his in different plant parts**

**(a)** Immunoblotting analysis of samples collected 8 dpi, using anti-PPV (top) and anti-his (bottom) antibodies. Lanes 1 – inoculated leaf, lanes 2 – non-inoculated leaf, lanes 3 – top apex, lanes 4 – stem, lanes 5 – root, lane 6 – molecular weight marker (values on the right). The arrows show the positions of intact capsid protein and P1-his, respectively. The samples were denatured after clarification (left from the marker), or before clarification (right from the marker). **(b)** Immunoblotting analysis of root samples collected during early stage of infection by pIC-PPV-Rec-P1His using anti-his antibodies. Lane 0 – healthy control, lanes 1-10 – collection time 1-10 dpi, lane 11 – molecular weight marker (values on the right). The samples were denatured before clarification.

been anticipated. It influences the symptom manifestation (Nagyová *et al*., 2012; Maliogka *et al*., 2012) and host specificity (Salvador *et al*., 2008), however, only speculations about the mode of its action *in vivo* are still at disposal. There are indicia that it cooperates with other potyviral protein (HC-pro) in suppression of host gene silencing defense (Valli *et al*., 2006) or interacts with ribosomes to stimulate viral protein synthesis at the expense of the host own one (Martínez and Darós, 2014). Knowledge about expression kinetics and localisation of P1 *in vivo* contributes to the comprehensive image of its real role during potyviral infection.

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