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Transient expression of the influenza A virus PB1-F2 protein using a plum pox virus-based vector in *Nicotiana benthamiana*

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Summary. – PB1-F2 protein of influenza A virus (IAV) was cloned in a plum pox virus (PPV) genome-based vector and attempts to express it in biolistically transfected *Nicotiana benthamiana* plants were performed. The vector-insert construct replicated in infected plants properly and was stable during repeated passage by mechanical inoculation, as demonstrated by disease symptoms and immunoblot detection of PPV capsid protein, while PB1-F2-specific band was more faint. We showed that it was due its low solubility. Modification of sample preparation (denaturation/solubilization preceding the centrifugation of cell debris) led to substantial signal enhancement. Maximal level of PB1-F2 expression in plants was observed 12 days post inoculation (dpi). Only 1% SDS properly solubilized the protein, other detergents were much less efficient. Solubilization with 8M urea released approximately 50% of PB1-F2 from the plant tissues, thus the treatment with this removable chaotropic agent may be a good starting point for the purification of the protein for eventual functional studies in the future.

Keywords: viral vector; potyvirus; biolistics; heterologous expression

PB1-F2 protein is a pathogenicity determinant of influenza A virus (IAV) translated from an alternative (+1) reading frame in the PB1 gene (Chen *et al*., 2001). It has been shown to target mitochondrial membrane and induce cell death via interactions with mitochondrial membrane proteins (Gibbs *et al*., 2003; Yamada *et al*., 2004; Zamarin *et al*., 2005). Moreover, it plays a role in the IFN type I response modulation (Goffic *et al*., 2010; Conenello *et al*., 2011; Dudek *et al*., 2011; Le Varga *et al*., 2011). Single mutation in PB1-F2 increased virulence of the IAV (Conenello *et al*., 2007). The mechanism of action is so far unknown, however, interaction of PB1-F2 with viral RNA polymerase subunit PB1 is proposed to be involved in this process (Mazur *et al*., 2008; Košík *et al*., 2011). *In vivo*, PB1-F2 is very unstable and prone to rapid degradation under physiological conditions (Chen *et al*., 2001; Schmolke *et al*., 2011).

Plum pox virus (PPV) belongs to the genus *Potyvirus* (the family *Potyviridae*). It has a (+)ssRNA genome with the length of nearly 10 kb, expression of which is based on the polyprotein processing strategy – the product of a single ORF is co- and post-translationally digested by virus-encoded proteases (Salvador *et al*., 2006). Consequently, all viral proteins are produced in equimolar amounts and their actual activity in infected cells depends on their stability (degradation half-life) or modification (e. g. deposition in inclusion bodies).

Insertion of exogenous genes between regions coding for particular viral proteins in the infectious PPV cDNA may lead to production of foreign proteins in infected plants. Several examples of biofarming using potyviral expression vectors have been published (Komarova *et al*., 2010). Recently, a full-length infectious cDNA clone of the strain PPV-Rec was constructed and adopted as biologically safe

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Abbreviations: dpi = days post inoculation; IAV = influenza A virus; PPV = plum pox virus

viral vector for transient expression (Kamencayová and Šubr, 2012; Predajňa *et al*., 2012). Here we present our attempts to use this PPV-based vector for stable expression of IAV PB1-F2 in plants.

We used the PPV-Rec-based vector pBOR-VT (Kamencayová and Šubr, 2012) constructed by combining the PPV-Rec infectious full-length cDNA clone (Predajňa *et al*., 2012) and previously prepared vector PPV-NK (Fernández-Fernández *et al*., 2001). The complete PB1-F2-coding sequence (261 bp) was amplified from the previously prepared construct pTriEX4 PB1-F2 (Košík *et al*., 2011) using EX Taq DNA polymerase (TaKaRa) and primers including *Eag*I and *Kpn*I linkers, respectively (underlined in the sequences): AACGGCCGGATGGGACAGGAACAGGATAC, AAGGTACCCTCGAGTTTGCTGAACAACC. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, 40 cycles of 94°C/15 sec, 58°C/30 sec, 72°C/1 min, and final extension at 72°C for 10 min. The PCR product was directly cloned into pGEM-Teasy (Promega), followed by *Eag*I/*Kpn*I cleavage and recloning in frame in pBOR-VT. FastDigest restriction enzymes (Fermentas) were used for DNA cleavage. The resulting plasmid was transformed and multiplied in *Escherichia coli* JM109 and purified by PureYield Plasmid Miniprep System (Promega). For the clone analysis, colony PCR was applied with the primer pair NCuniFor/NCuniRev (Predajňa *et al*., 2012) spanning the PPV NIb-CP border, where the foreign gene was inserted. The conditions were identical as described above except the elongation time was 1.5 min in each cycle. The amplimers were sequence-verified and corresponding correct plasmid clones were used for plant transfection. Two weeks old *N. benthamiana* plants were transfected biolistically by an air-gun technique as described before (Predajňa *et al*., 2010) and maintained under insect-free conditions at 23°C and photoperiod 14/10 hr. PPV symptoms were visually evaluated 10–15 dpi, crude leaf sap samples were analyzed by SDS-PAGE in 15% polyacrylamide gel and immunoblotting with anti-PPV polyclonal antibody (Šubr and Matisová, 1999) and anti-PB1-F2 monoclonal antibody AG55, respectively (Krejnusová *et al*., 2009). Total protein from plant tissues was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's recommendation. For further analysis, total RNA from infected plants was isolated using the NucleoSpin RNA Plant Kit (Macherey-Nagel), random hexamer-primed cDNA synthesis was carried out using AMV reverse transcriptase (Promega), and PCR with primers NCuniFor/NCuniRev was performed as described above.

From the ten clones analyzed by colony PCR, three harbored no insert in the pBOR-VT and three showed sequence defects at the cloning sites resulting in incorrect frame of the inserted fragment (data not shown). *N. benthamiana* plants were transfected by each of the four clones with correct sequence of the analyzed region. Following transfection,

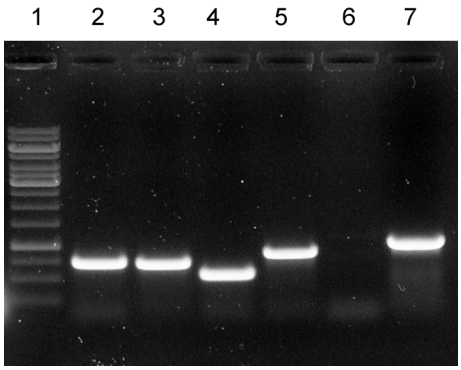

Fig. 1
RT-PCR analysis of plants transfected by four pBOR-VT-PB1-F2 clones
1 kb DNA Ladder (lane1), clone 1 (lane 2), clone 2 (lane 3), clone 3 (lane 4), clone 4 (lane 5), negative PCR control (lane 6), positive PCR control – plasmid (lane 7).

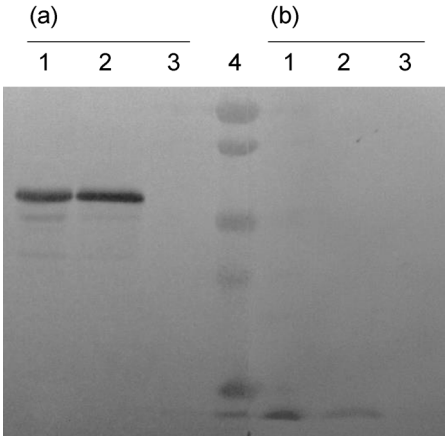

Fig. 2
Immunoblotting analysis of plants transfected by pBOR-VT-PB1-F2 clone 4
Samples collected 7 dpi (lanes 1) or 14 dpi (lanes 2), healthy control (lanes 3), prestained protein MW marker (lane 4). Immunodetection by anti-PPV (a) or anti-PB1-F2 (b) antibodies.

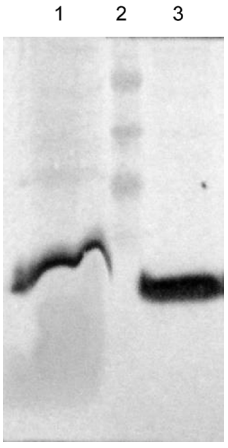

Fig. 3
Comparison of different sample preparation for PB1-F2 detection
Immunoblot using anti-PB1-F2 antibody of samples from plants transfected by pBOR-VT-PB1-F2 10 dpi. Crude sap boiled with SDS prior centrifugation (lane 1), prestained protein MW marker (lane 2), total protein isolated by TRIzol method (lane 3).

three of them showed typical symptoms of PPV infection of the plant, including mosaic and leaf deformation. Symptom manifestation correlated well with the immunoblotting analysis of the crude plant sap using anti-PPV antibody. However, only one sample (clone 4) gave a weak signal with anti-PB1-F2 antibody. RT-PCR and sequence analysis from total RNA obtained from *N. benthamiana* confirmed the presence of PB1-F2 sequence only in the clone 4-infected plants. The other clones obviously lost the insert during early multiplication *in planta*, in the case of clone 3 the deletion included part of the viral CP gene, leading to the loss of infectivity (Fig. 1).

The clone 4 was stable in plants during several passages by mechanical inoculation using the infectious plant sap. PB1-F2 concentration detected by immunoblotting, however, remained low and further decreased with the time after infection (Fig. 2). Sampling in very early infection stages (1–4 dpi) showed similar results. It has been demonstrated that PB1-F2 tends to form amyloid fibers in membrane environments (Chevalier *et al.*, 2010). We, therefore, presumed that the majority of produced protein could be part of the insoluble fraction and sedimented together with the cell debris during sample preparation. To prevent this possibility, we added the SDS-containing sample buffer (Laemmli, 1970) directly to the homogenized tissue, vortexed thoroughly and boiled in the water bath for ten minutes. Thereafter the debris was removed by centrifugation and the supernatant analyzed by SDS-PAGE and immunoblotting. Samples prepared by this modified procedure gave notably stronger signal (Fig. 3). In the light of this finding, the aparent concentration drop of PB1-F2 in prolonged infection reflected rather growing insolubility of the protein during its accumulation in the cells than its proteolytic degradation. Excess of chlorophyll in modified samples sometimes partially interferred with detection of the relatively small PB1-F2 (10.5 kDa), even in 15% polyacrylamide gels. This problem could be fixed by sample preparation using TRIzol reagent (Fig. 3).

Maximal level of PB1-F2 expression in plants was observed 12 dpi, its concentration later moderately dropped (data not shown). Our attempts to purify PB1-F2 under native conditions (without SDS) remained, however, unsuccessful. Application of other agents like Triton X-100, CHAPS or guanidinium-Cl was not successful, expressed PB1-F2 resisted to these solubilization attempts. Only 8M urea combined with ultrasonication released approximately 50 % of the protein from the plant tissues. Urea has been applied also for solubilization of PB1-F2 expressed in *E. coli* (Chevalier *et al.*, 2010).

Here we report the first attempt to express IVA PB1-F2 in *N. benthamiana* using a plant viral vector. Although stable multiplication of the vector pBOR-VT-PB1-F2 in plants was recorded, leading to constitutive transient expression of the desired product, insoluble form of the protein prevented at the moment its efficient extraction and purification for further studies. Denatured recombinant PB1-F2 obtained in sufficient quantity may be applied as an antigen for immunization and production of specific antibodies. Moreover, pilot extraction experiments with urea let us presume the possibility of PB1-F2 purification from the crude soluble fraction in presence of chaotropic agents. It remains a challenge for the future to optimize the conditions of the protein purification. As urea can be removed at the end of the procedure, e.g. by dialysis, it is likely that a fully functional renatured product can be obtained.

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