DOI: 10.1007/s10535-012-0130-2 BIOLOGIA PLANTARUM 56 (4): 775-779, 2012

BRIEF COMMUNICATION

The influence of the N- and C- terminal modifications of *Potato virus X* coat protein on virus properties

H. HOFFMEISTEROVA[1,2], T. MORAVEC[1], H. PLCHOVA[1], J. FOLWARCZNA[1,3] and N. CEROVSKA[1]*

Laboratory of Virology, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1a, CZ-16000 Prague 6, Czech Republic[1]
Department of Crop Protection, Czech University of Life Sciences Prague, Kamycka 129, CZ-16521 Prague 6, Czech Republic[2]
Department of Biochemistry, Faculty of Science, Charles University in Prague, Albertov 6, CZ-12843 Prague 2, Czech Republic[3]

Abstract

The *Potato virus X* (PVX)-based vector was used for the construction of N- and C-terminally modified PVX coat protein (XCP) chimeras. N-terminal XCP modifications do not influence the viral life cycle, whereas the simple XCP C-terminal fusion impedes the viral replication. We designed several C-terminally modified XCP chimeras and tested their viabilities in various *Nicotiana benthamiana* genotypes. Our results showed the negative impact of 3´-terminal modification of XCP on the chimera´s life cycle. To ensure chimeric constructs stability, the second copy of the last 60 nucleotides of XCP followed by the 3´-untranslated region (UTR) was added downstream of the recombinant sequence. Simultaneously, the first copy of the last 60 nucleotides of XCP was mutated in order to prevent recombination between the two identical sequences. The movement protein of *Tobacco mosaic virus* expressed in transgenic *N. benthamiana* plants positively affected the cell-to-cell spread of C-terminally modified XCP chimeras.

Additional key words: chimeric coat protein, expression of recombinant protein, *Nicotiana benthamiana,* terminal fusion.

Potato virus X (PVX) is a type member of the genus *Potexvirus*, family *Flexiviridae.* PVX is frequently used as a model to study the molecular mechanisms controlling virus replication and movement (Batten *et al*. 2003, Atabekov *et al*. 2007, Verchot-Lubicz *et al.* 2007). PVX forms filamentous particles of about 515 nm in length and 13 nm in diameter (Sonenberg *et al.* 1978). The particle consists of a 6.4 kb long positive sense single-stranded genomic RNA which is capped and polyadenylated. The RNA contains a 84 nucleotide (nt) long 5´-untranslated region (UTR) followed by five open reading frames coding for the 165 kDa replicase, triple gene block proteins TGBp1, TGBp2 and TGBp3 involved in virus movement, a 25 kDa coat protein (CP) and the 72 nt long 3´-UTR (Huisman *et al.* 1988, Skryabin *et al.* 1988). The approximately 1 300 copies of the PVX CP (XCP) encapsidate the viral RNA in such way that their N-termini are exposed on the virus surface (Baratova *et al.* 1992). XCP is also necessary for cell-to-cell and systemic movement in the host plants (Chapman *et al.* 1992a, Santa Cruz *et al.* 1996, 1998, Morozov and Solovyev 2003, Verchot-Lubicz 2005). The recent model of PVX cell-to-cell movement describes the non-virion ribonucleoprotein (RNP), a complex of viral RNA, XCP and TGBp1 formed into so called single-tailed particles (STPs), an agent trafficking the plasmodesmata (Lough *et al.* 1998, 2000, Karpova *et al.* 2006, Verchot-Lubicz *et al.* 2007). Furthermore, in the transient complementation experiments it was demonstrated that XCP has at least two movement-associated functions. The movement

Received 21 July 2011, *accepted* 7 December 2011.
Abbreviations: CP - coat protein; HPV16 - *Human papillomavirus* type 16; L2 - epitope derived from the L2 CP of the HPV16 (aa 108 - 120); L2-XCP - N-terminally modified XCP; MP - movement protein; PVX - *Potato virus X*; RNP - ribonucleoprotein; STPs - single-tailed particles; TSP - total soluble protein; UTR - untranslated region; VLPs - virus-like particles; XCP - CP of the PVX; XCP-L2, XCP-L2-Δ, XCP-L2-mut - XCP with different C-terminal modifications.
Acknowledgements: This research was supported by the grant 521/09/1525 of the Grant Agency of the Czech Republic and the fellowship CZU 21180/1312/3126 from the Czech University of Life Sciences, Prague.
* Corresponding author; fax: (+420) 224 310113, e-mail: cerovska@ueb.cas.cz

function of XCP can be, however, complemented in the presence of the movement protein of *Tobacco mosaic virus* (MP TMV) (Chapman *et al.* 1992a, Tamai and Meshi 2001).

It is known that the XCP N-terminal genetic fusions of recombinant sequences do not impede the viral viability and particle assembly in principle. Therefore the PVX is broadly utilized as highly ordered, multivalent carrier of recombinant proteins/epitopes for pharmaceutical or industrial use (Chapman *et al.* 1992b, Baulcombe *et al.* 1995, Toth *et al.* 2001, Porta *et al.* 2002, Cerovska *et al.* 2004, 2008, Cañizares *et al.* 2005, Uhde-Holzem *et al.* 2007, Hoffmeisterova *et al.* 2008, Plchova *et al.* 2011). The modification of the XCP C-terminal part is complicated because the region of the last 60 nt of XCP together with the 3´-UTR plays a role both in the virus replication and in particle assembly (Chapman *et al.* 1992a, Forster *et al.* 1992, Fedorkin *et al.* 2001, Pillai-Nair *et al.* 2003). However, the first successful expression of XCP modified by the C-terminally fused E7 oncoprotein from *Human papillomavirus* type 16 (HPV16) was recently published. The construct was based on PVX in which downstream of the stop codone of modified XCP the second copy of a last 60 nt of XCP followed by the 3´-UTR was present. Unfortunately, this modified XCP did not form virus-like particles (VLPs; Plchova *et al.* 2011).

In the present study, all used constructs were derived from the plant viral vector pGR106 based on PVX (kindly provided by D.C. Baulcombe, The Sainsbury Laboratory, Norwich, United Kingdom). Briefly, the XCP wild type (wt) coding sequence was amplified by PCR using primers carrying the suitable restriction sites to be easily cloned into pMPM-A4Ω (Mayer 1995). The coding sequence of the epitope derived from the HPV16 L2 (amino acids 108 - 120: LVEETSFIDAGAP; Kawana *et al.* 1998) was prepared by annealing of oligonucleotides coding also for appropriate restriction sites. The sequences were then fused with either the 3´- or 5´-terminus of the XCP sequence in the bacterial expression vector pMPM-A4Ω using relevant restriction sites. The resulting constructs were then subcloned into *pGEM-T Easy* (*Promega*, Madison, WI, USA) to add suitable flanking cloning sites for subsequent cohesive-ends cloning into the full-length clone pGR106 from which the wt XCP coding sequence was removed. In this way the additional copy of subgenomic CP promoter and the XCP originally present in pGR106 was replaced by the chimeric constructs L2-XCP and XCP-L2 (Fig. 1). The constructs XCP-L2-Δ and XCP-L2-mut were prepared by SOE-PCR (Horton 1995). Briefly, in the first round of PCR two amplicons were prepared using pGR106 carrying XCP-L2 as a template and two flanking primers and two central primers. The PCR products were gel-purified, mixed together and used as a template for second round of PCR in which only flanking primers were used. The final product was subcloned into pJET1.2/blunt (*Fermentas Germany*, St. Leon-Rot, Germany; Fig. 1) and subsequently into pGR106. All constructs were sequenced.

Agrobacterium tumefaciens strain GV3101 (*Biogen*, Prague, Czech Republic) was transformed by the freeze-thaw method (Chen *et al.* 1994). Bacteria containing the plasmids were selected on Luria broth (LB)-agar plates containing 30 μg cm^{-3} kanamycin and suspensions for infiltration were grown in LB medium with kanamycin at 28 °C to absorbance (A_{550}) of 0.5. *Nicotiana benthamiana* and transgenic *N. benthamiana* plants expressing the movement protein of *Tobacco mosaic virus* (original designation Nb3H, for clarity renamed as *N. benthamiana*

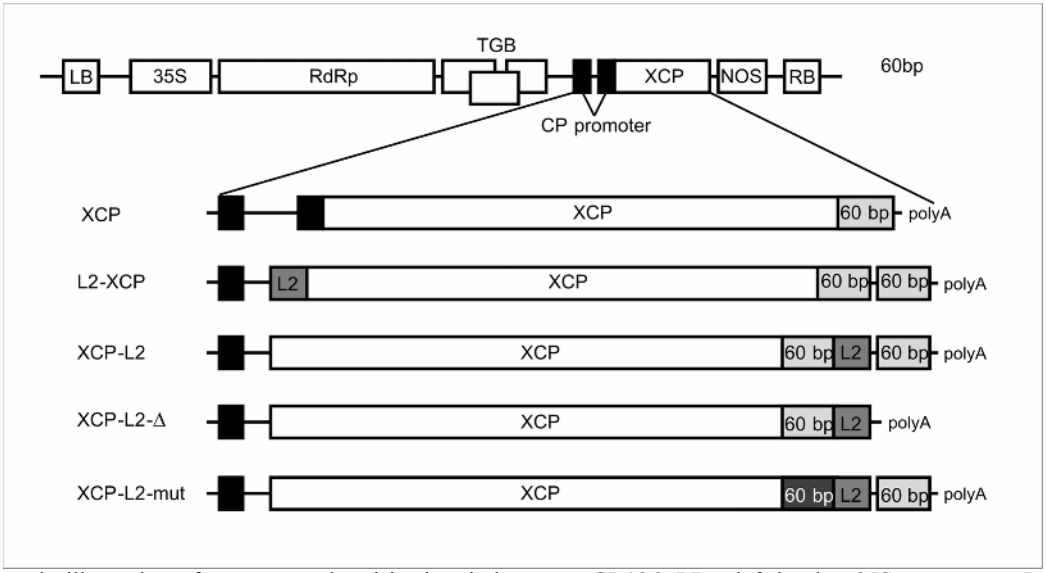

Fig. 1. Schematic illustration of constructs placed in the viral vector pGR106. LB - left border, 35S - promoter, RdRp - RNA-dependent RNA polymerase, TGB - triple gene block, L2 - HPV16 L2 epitope, XCP - PVX CP, NOS - nopalin synthase terminator, RB - right border. Epitope L2 (*middle gray*), the last 60 bp of XCP RNA (*light grey*) and the sequence of the last 60 bp of XCP RNA which is mutated without amino acids altering (*dark grey*) are indicated.

MP throughout this paper; provided by Dr. R.N. Beachy, Donald Danforth Plant Science Center, St. Louis, USA) were infiltrated with appropriate cultures of *A. tumefaciens* using syringe without needle (Schöb *et al.* 1997).

The samples were prepared from inoculated leaves, diluted to the same level of total soluble proteins (TSPs; D_C *Protein Assay*, *Bio-Rad*, Prague, Czech Republic) in the standard sample buffer containing extra 6 M urea and 40 µg of TSP was loaded onto 5 % stacking and 12 % resolving gel to perform sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). Separated proteins were electroblotted onto a nitrocellulose membrane (*Protran BA83*, *Whatman*, Maidstone, UK) according to Cerovska *et al.* (2004).

The L2-XCP chimera was expressed in both hosts in a relatively high amount (0.8 - 2.0 % of TSP in comparison with 4.0 - 8.0 % in the case of unmodified XCP) with the slightly lower electrophoretic mobility than the wt XCP. No protein was detected in leaves inoculated with XCP-L2-Δ using the SDS-PAGE/WB analysis. In the leaves of both *Nicotiana* genotypes inoculated with XCP-L2 was detected one unique band of comparable expression level and also electrophoretic mobility as XCP (Fig. 2*A*). Therefore, from the leaves inoculated with the construct XCP-L2 was extracted total RNA by the *TRI Reagent* ® (*MRC*, Ontario, Canada) followed by DNAse I treatment (*Ambion*, Austin, TX, USA) used according to the manufacturer's instructions and PCR amplification using specific primers was performed. The PCR product was identified by nucleotide sequencing as XCP, therefore the evidence of excision of the L2 epitope flanked by homologous 60 nt sequences was supposed. Next, the construct XCP-L2-mut in which the first copy of last 60 nt of XCP was mutated without change of amino acid (aa) sequence to prevent the supposed recombinantion was designed (Fig. 1). In inoculated leaves of non-transgenic *N. benthamiana* was detected a protein with the same eletrophoretic mobility as XCP whereas in inoculated leaves of *N. benthamiana* MP was the electrophoretic mobility of detected protein slightly lower (Fig. 2*A*). After isolation of total RNA from *N. benthamiana* MP inoculated leaves and subsequent RT and PCR completion, the PCR product was identified as XCP-L2-mut, so there is the high probability that the immunodetected protein was XCP-L2. The expression level of XCP-L2 protein was 0.1 - 1.0 % of TSP.

Purification of XCP and its chimeras from plant tissues was performed as described by Cerovska *et al.* (1991). The isolation of the chimeric protein L2-XCP was performed and subsequent immunogold staining according to Beesley *et al.* (1984) followed by the transmission electron microscopy (TEM; *Jeol*, Tokyo, Japan; situated in Biology Centre of the Academy of Sciences, České Budějovice, Czech Republic; kindly performed by Dr. Helena Synkova, IEB CAS, Prague) proved the VLPs formation. No successful purification of the C-terminally modified chimera was achieved suggesting the instability of chimeric particles built from XCP-L2 protein.

This fact was supported by results obtained using following methods: the density of the protein expressed from the construct XCP-L2-mut in the sucrose gradient was compared to the mobility of XCP and L2-XCP. Briefly, the crude plant extracts were loaded onto sucrose gradient (10 - 60 % sucrose in 0.05 M Na-K phosphate buffer, pH 7.3) and centrifugated in 5-cm^3 cuvettes at 42 000 *g* for 1.5 h (*SW55Ti* rotor, *L7-55* ultracentrifuge, *Beckman,* Coulter, CA, USA). The 1-cm^3 fractions were collected from the top and the pellet was resuspended in 1 cm^3 of 0.05 M Na-K phosphate buffer, pH 7.3. All fractions were analyzed by SDS-PAGE and immuno-blotting. The L2-XCP protein was detected in the same fractions as XCP whereas the chimera protein XCP-L2 expressed from the construct XCP-L2-mut remained on the top of the cuvette (Fig. 2*B*).

Furthermore, no viral particles were observed in crude extract containing protein XCP-L2 using immunogold labeling and negative staining according to a standard

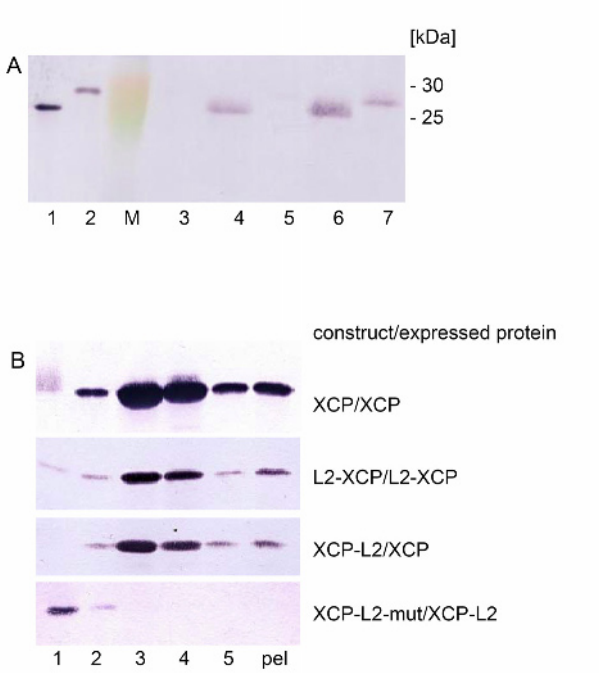

Fig. 2. SDS-PAGE/WB analysis of the plant samples using the polyclonal anti-XCP antibodies (*A*). Samples were prepared from *N. benthamiana* MP leaves inoculated with construct XCP (*lane* 4; 20 × diluted), XCP-L2-Δ (*lane* 5), XCP-L2 (*lane* 6) and XCP-L2-mut (*lane* 7). As a control leaves of non-inoculated plants (*lane* 3) and purified XCP (*lane* 1; 60 ng) and L2-XCP (*lane* 2; 120 ng) were used. SDS-PAGE/WB analysis of the fractions obtained after ultracentrifugation of crude plant extracts in the sucrose gradient (*B*). The crude extracts were prepared from *N. benthamiana* MP leaves inoculated with indicated constructs expressing appropriated proteins. After ultracentrifugation five 1 cm^3-fractions were collected from the top to the botom of the cuvette (fractions 1 - 5) and the pellet (pel) was resuspended.

procedure (Beesley *et al.* 1984) followed by the TEM in comparison with extract containing the L2-XCP.

Moreover, the XCP and XCP-L2-mut coding sequences were amplified from inoculated *N. benthamiana* MP leaves using either total RNA as a template or using immunocaptured virus particles. The immunocapture RT-PCR procedure was performed essentially as described in Cerovska *et al.* (2004) using anti-XCP antibodies (*Bioreba,* Reinach, Switzerland). In both cases the cDNA was synthesized using anchored oligo-dT and RNaseH minus MuMLV reverse transcriptase (*Promega*) followed by the PCR using specific primers. The band corresponding to the chimera XCP-L2-mut was only amplified when total RNA was used as a template for cDNA synthesis; in samples in which immunocapture was used to prepare the RNA template, the PCR failed to amplify the expected band.

It was confirmed that the last 60 bp of the XCP directly followed by the 3´-UTR are essential for the completion of the viral life-cycle because no expressed protein reacting with the anti-XCP antibodies was detected in inoculated leaves of hosts infected with the construct XCP-L2-Δ. It was also confirmed that the cell-to-cell movement of *Potato virus X* can be compensated by the TMV MP as published previously (Fedorkin *et al.* 2001, Tamai *et al.* 2003). However, the C-terminus of the XCP probably plays a role in the particle assembly and assembly-deficient virus failed to move into systemic leaves in *N. benthamiana* MP. Furthermore, in both used hosts exists a strong selection pressure causing the recovery of C-terminally modified XCP into the wt virus and its subsequent rapid multiplication without recombinant sequences. Such event occurred in both genotypes of plants inoculated with the construct XCP-L2 and also in the non-transgenic *N. benthamiana* plants inoculated with the construct XCP-L2-mut although in this construct one copy of the homologous sequence was mutated to prevent the recombination. The C-terminally modified XCP was expressed only in leaves of *N. benthamiana* MP inoculated with XCP-L2-mut. Although the chosen L2 epitope did not interfere with the VLPs formation if fused to the N-terminus of XCP, the C-terminally modified XCP-L2 chimera did not form VLPs.

In conclusion, the presence of a short recombinant peptide situated on the XCP N-terminus (construct L2-XCP) did not basically interfere with the viral life cycle because the cell-to-cell and long-distance movement of the chimera as well as the ability to form chimeric virions was confirmed. In the case of the simple XCP C-terminal fusion (construct XCP-L2-Δ) no target protein was detected. The presence of a second copy of the last 60 bp of XCP placed downstream of the stop codone of the target protein (construct XCP-L2) led to the reversion of the chimera to the wt virus by homologous recombination between duplicated 60 bp sequences in both used hosts. The C-terminal recombinant fusion protein was expressed using the construct XCP-L2-mut in inoculated leaves of transgenic *N. benthamiana* expressing the MP TMV whereas in non-transgenic hosts the reversion of the chimera into the wt virus occurred. The ability of the XCP-L2 protein to form VLPs was not confirmed although it was supposed that in the case of epitope-fusion to XCP the steric hindering should be minimized. Therefore there is a great need of work in this area of research, especially in terms of cell-to-cell and long-distance movement of C-terminally modified XCP chimeras and their ability to form VLPs. These information could help to improve the properties of viral vectors based on PVX.

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