Detection of 6K1 as a mature protein of 6 kDa in plum pox virus-infected *Nicotiana benthamiana*

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The RNA genome of *Plum pox virus* (PPV) encodes one large polyprotein that is subsequently cleaved into mature viral proteins. One of the products of proteolytic processing, the 6K1 protein, has not yet been identified *in vivo* for any member of the genus Potyvirus. In this study, 6K1-specific polyclonal antisera was raised against PPV 6K1 expressed in *Escherichia coli* as a translational fusion with the N terminus of avian troponin C and an unusual metal-binding cluster of troponin T-1. For detection of 6K1 *in vivo*, a pPPV-H6K1-NAT infectious clone was constructed, enabling concentration of histidine-tagged 6K1 by affinity chromatography. Affinity-purified 6K1 was detected in locally infected *Nicotiana benthamiana* leaves at 4, 7 and 14 days post-inoculation (d.p.i.) and, in addition, in systemically infected leaves at 14 d.p.i., 6K1 was detected exclusively as a protein of 6 kDa and no polyprotein precursors were identified with the raised anti-6K1 antiserum.

In order to clarify whether 6K1 is produced *in vivo* in potyvirus-infected cells, we raised an antiserum directed against bacterially expressed 6K1 of a non-aphid-transmissible isolate of PPV (PPV-NAT) (Maiss et al., 1989). To facilitate the detection of 6K1 from PPV-NAT-infected plants, we engineered a full-length PPV-NAT infectious clone (Maiss et al., 1992), pPPV-H6K1-NAT, enabling the concentration and purification of histidine-tagged 6K1 from infected tissue by affinity chromatography. Finally, we report the detection of 6K1 as a mature protein of 6 kDa in affinity-purified lysates of PPV-H6K1-NAT-infected *Nicotiana benthamiana*.

The coding sequence of PPV-NAT-6K1, corresponding to aa 1117–1168 in the PPV polyprotein, was PCR-amplified from plasmid pPPV-NAT (Maiss et al., 1992) by using oligonucleotide primers 5'-AACCGCCCATATGGAATGGCAGTAAGAGAGACTCAC-3' and 5'-AGCCC GGATCCTA CTGATGATGAACAG-3' (restriction sites underlined in primer sequences) and inserted into the bacterial expression vector pPEPTIDE1 (MoBiTec) in frame with the N terminus of avian muscle troponin C (TnC) and an unusual metal-binding cluster of avian muscle troponin T-1 (Tx). The resulting pPEPTIDE-6K1 was transformed into *Escherichia coli* strain BL21(DE3)-RIL (Invitrogen) and crude cell extracts of the transformed bacteria were tested for the expression of TnC-Tx-6K1 fusion protein by SDS-PAGE analysis. Induction of cells with IPTG resulted in the expression of a protein with the expected molecular mass of TnC-Tx-6K1 (Fig. 1a). As the metal-binding cluster Tx, Spetz & Valkonen, 2004). In contrast to 6K2, little attention has been paid to 6K1 and it is the last potyviral product that remains to be identified as a mature protein *in vivo*.

Identification of specific amino acid motifs within the polyprotein is used to predict the genetic map of newly sequenced potyviruses. Most potyviruses sequenced to date are predicted to encode ten mature viral proteins, namely P1, HC-Pro, P3, a protein of 6 kDa (6K1), cylindrical inclusion protein (CI), a second protein of 6 kDa (6K2), genome-linked protein (VPg), the protease part of Nla (Nla-Pro), nuclear inclusion protein b (NIb) and coat protein (CP). Most mature proteins have been detected in potyvirus-infected cells for at least one representative of the genus *Potyvirus* (Rodriguez-Cerezo & Shaw, 1991). One of the proteins of 6 kDa, 6K2, has been immunodetected in *Tobacco etch virus* (TEV)-infected cells and its function has subsequently been studied (Kekarainen et al., 2002; Klein et al., 1994; Léonard et al., 2004; Martin & Gélie, 1997; Merits et al., 2002; Rajamäki & Valkonen, 1999, 2002; Restrepo-Hartwig & Carrington, 1994; Schaad et al., 1997; Spetz & Valkonen, 2004). In contrast to 6K2, little attention has been paid to 6K1 and it is the last potyviral product that remains to be identified as a mature protein *in vivo*.

*Plum pox virus* is a member of the genus *Potyvirus* within the plant-infecting family *Potyviridae*. The potyviral genome consists of single-stranded RNA of positive polarity and contains one large open reading frame (ORF). Translation of this ORF generates a polyprotein precursor of ~350 kDa that is subsequently processed by three virus-encoded proteases, giving rise to intermediates or final products of proteolytic processing (Adams et al., 2005; Lopez-Moya et al., 2000; Merits et al., 2002; Riechmann et al., 1992; Urcuqui-Inchima et al., 2001). Two proteases, P1 and helper-component protease (HC-Pro), catalyse cleavage at their own respective C termini. The third viral proteolytic activity resides within the C terminus of nuclear inclusion protein a (Nia) and mediates most cleavage reactions – both *in cis* and *in trans* – at cleavage sites defined by conserved heptapeptide sequences (Adams et al., 2005).
present in the expressed fusion protein, was also reported to bind to Ni\(^{2+}\) (Jin & Smillie, 1994), TnC–Tx–6K1 was purified by affinity chromatography on Ni-NTA columns (Qiagen) following the manufacturer's instructions. Eluates containing TnC–Tx–6K1 protein (Fig. 1a) were dialysed against 0.5 × PBS and used for the immunization of rabbits. Prior to application in immunoblot analyses, the anti-6K1 antiserum and the anti-CP antiserum (Riedel et al., 1998) were purified by using protein A columns (Pierce).

To validate that the antiserum raised against TnC–Tx–6K1 was directed against 6K1 and did not react exclusively with the TnC–Tx part of the antigen, 6K1 was expressed bacterially as a translational fusion with histidine-tagged dihydrofolate reductase (DHFR–H6K1). The 6K1 gene of PPV-NAT was cloned into expression vector pQE40 (Qiagen) and DHFR–H6K1 or DHFR–H was expressed in E. coli strain SG13009 (pREP4) (Qiagen; Gottesman et al., 1981) following the manufacturer’s instructions. Expression of proteins was monitored by subjecting lysates of induced bacteria to immunoblot analysis with anti-His antibody. Immunoblots were optimized for detection of small and basic proteins. Briefly, proteins present in cell lysates were separated by Tricine/SDS-PAGE (Schägger & von Jagow, 1987) and blotted onto nitrocellulose membranes (pore size, 0.2 µm) in alkaline buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11), 20 % (v/v) 2-propanol]. Blots were probed with primary antibodies [anti-6K1 antiserum; His-tag mAb (Novagen); anti-CP antiserum] and respective species-specific secondary antibodies: horseradish peroxidase-conjugated anti-rabbit F(ab’)\(_2\) fragments (Jackson Immunoresearch), alkaline phosphatase-conjugated anti-mouse IgG (Loewe), alkaline phosphatase-conjugated anti-rabbit IgG (Sigma); followed by the detection of secondary antibodies by enhanced chemiluminescence or Fast red TR staining.

Both DHFR–H6K1 and DHFR–H were expressed and present on blots in equal amounts, as verified by Ponceau S staining (data not shown) and anti-His immunoblot analysis (Fig. 1b). Still, anti-6K1 antiserum recognized DHFR–H6K1, but not DHFR–H, proving its specificity for bacterially expressed 6K1 (Fig. 1b). Additionally, the sensitivity of the anti-6K1 antiserum was assessed by using Ni-affinity-purified DHFR–6K1 protein. Anti-6K1 immunoblot analysis was capable of detecting as little as 1 ng purified DHFR–6K1 protein, which corresponds to the detection limit of anti-CP immunoblot analyses used in this study (data not shown). Therefore, the raised anti-6K1 antiserum proved to be both specific and sensitive for bacterially expressed 6K1 and was further used for the detection of 6K1 from plants.

In the first attempts, the antiserum failed to react with proteins in extracts of PPV-NAT-infected N. benthamiana. In order to improve the detection procedure, we applied a strategy similar to that described previously for the detection of 6K2 of TEV (Restrepo-Hartwig & Carrington, 1994). Henceforth, pPPV-H6K1-NAT was constructed, enabling Ni-affinity purification of histidine-tagged 6K1 produced in planta. To obtain PPV-H6K1-NAT with codons for seven histidine residues between the first and second codons of 6K1 (Fig. 2), a recombinant fragment was generated in a splicing by overlap-extension PCR (Horton et al., 1989), using primers 5’-TGACTTAGATTCTCAGTACTCAGAC-3’, 5’-CTCTCTTATGGTGATGGTGATGGTGATGACTT-TGATGAAACAC-3’, 5’-CAAAGTCATCACCATCACATCACCATAAAGAGACTCAGAC-3’ and 5’-TCCAGCTCTGTATGAAACAC-3’, and subsequently cloned into the pPPV-NAT infectious clone.

To determine whether the insertion of the histidine tag had an influence on infectivity, pPPV-H6K1-NAT and, as a control, pPPV-NAT were introduced into N. benthamiana by particle bombardment (Gray et al., 1994). All of the ten N. benthamiana plants inoculated with pPPV-H6K1-NAT or pPPV-NAT, respectively, developed typical systemic mosaic symptoms. To determine whether the histidine tag was maintained in the viral genome in vivo, total RNA was extracted from N. benthamiana inoculated with pPPV-H6K1-NAT. A subsequently RT-PCR-amplified fragment (Menzel et al., 2002) was cloned and sequenced, covering the 3’ end of P3 (corresponding to the C-terminal 74 aa) and the entire coding sequence of 6K1. When compared with pPPV-NAT, no difference was detected in this region in
spite of the insertion of the His codons. Thus, the newly generated pPPV-H6K1-NAT clone was used as a tool to investigate the expression of 6K1.

Two leaves of each *N. benthamiana* plant (*n* = 15) were inoculated mechanically with equal amounts of plant sap derived either from pPPV-H6K1-NAT- or pPPV-NAT-inoculated *N. benthamiana*. Six inoculated leaves were harvested for each virus at 1, 2, 4, 7 and 14 days post-inoculation (d.p.i.), ground immediately in liquid nitrogen and subsequently stored at −80 °C until analysis. Prior to subjecting locally infected (l.i.) leaf material to affinity purification, CP levels of infected plants were analysed and compared for both viruses, using an aliquot (1/30) of the leaf powder in immunoblot analysis with anti-CP antisera. In addition, symptom expression was monitored over the time course of infection. Whereas first leaf symptoms appeared for both viruses at 8 d.p.i. and were indistinguishable from each other, PPV-H6K1-NAT-infected *N. benthamiana* appeared to be less stunted after a time period of 2–3 weeks. Immuno-blot analysis with anti-CP antiserum showed an initial delay of CP expression of PPV-H6K1-NAT in comparison to PPV-NAT (Fig. 3). Whereas CP of PPV-NAT was already clearly detectable at 1 d.p.i., PPV-H6K1-NAT-CP was not clearly visible until 4 d.p.i. Nevertheless, CP levels of both PPV-H6K1-NAT and PPV-NAT l.i. leaves accumulated to similar amounts within a period of 14 days, in a manner similar to that shown for TEV CP (Baunoch et al., 1991). The remaining plant material (7 g) of PPV-H6K1-NAT- and PPV-NAT-infected plants was subjected to Ni-affinity chromatography under denaturing conditions as described by Restrepo-Hartwig & Carrington (1994) and obtained eluates were subjected to immunoblot analyses. As shown in Fig. 3, a protein of ~6 kDa was detected specifically with the anti-6K1 antiserum in eluates of affinity-purified plant extracts of PPV-H6K1-NAT-infected *N. benthamiana*, but not in those of PPV-NAT-infected ones. Apparently, fully processed 6K1 was detected for the first time at 4 d.p.i. and increasing levels were detected at 7 and 14 d.p.i. A low 6K1 protein level in infected plants could be one reason for the need for affinity purification prior

![Fig. 2. Construction of pPPV-H6K1-NAT by insertion of a histidine tag into pPPV-NAT. Relevant elements of the PPV-NAT full-length clone are shown in boxes [35S promoter of *Cauliflower mosaic virus*, cDNA sequence of PPV-NAT and a polyadenylation p(A) signal]. The PPV ORF is enlarged and viral gene products are shown as boxes named accordingly. Conserved Nla-cleavage sites are indicated by arrows. Manipulation of the 6K1 coding region is visualized on the amino acid level. Inserted amino acids are shown in grey; resulting histidine-tagged 6K1 (H6K1) is shown in bold type. Flanking Nla-recognition sequences are underlined.](http://vir.sgmjournals.org/2383)

![Fig. 3. Immunoblot analyses of PPV-H6K1-NAT (H6K1)- or PPV-NAT (NAT)-inoculated *N. benthamiana* leaves at different d.p.i. Leaf material of locally infected (l.i.) or systemically infected (s.i.) *N. benthamiana* was submitted directly to immunoblot analysis with anti-CP antiserum (anti-CP) or affinity-purified prior to immunoblot analyses with anti-6K1 antiserum (anti-6K1) or anti-His antibody (anti-His). Scanned immunoblot images were adjusted in contrast and brightness with Adobe Photoshop Elements 2.0.](http://vir.sgmjournals.org/2383)
molecular mass (Fig. 3). As these were detected not only in PPV-H6K1-NAT+, but also in PPV-NAT-infected and mock-inoculated plants (Fig. 3; data not shown), they probably represent plant proteins with affinity to Ni/agarose.

In addition, 6K1 expression was determined in systemically infected (s.i.) leaves. Prior to analysis of 6K1 expression, the presence of virus was verified by RT-PCR (data not shown) and immunoblot analysis with anti-CP antiserum (Fig. 3). In addition to apparently full-length CP, two putative degradation products of CP were detected, probably due to the digestion of the N and C termini, which are exposed on the surface of the virus particle (Allison et al., 1985; Hiebert et al., 1984; Shukla & Ward, 1989; Shukla et al., 1988). These degradation products were also detected at 14 d.p.i. in l.i. leaf material. Leaf material (30 g) for Ni-affinity purification was treated and analysed as described for l.i. leaves. As in l.i. leaf material, 6K1 was identified as a mature protein and no proteins of higher molecular mass were detected specifically with anti-6K1 antiserum. Additionally, and probably due to increased amounts of affinity-purified leaf material, 6K1 was identified with anti-His antibody when maximum amounts of eluate were loaded on an SDS/polyacrylamide gel (Fig. 3).

Taken together, PPV 6K1 was detected as a protein of 6 kDa in both locally and systemically infected N. benthamiana leaves. To our knowledge, this is the first time that potyviral 6K1 has been identified as a mature protein in planta. Furthermore, 6K1 was not detected as part of polyprotein precursors in this study and it is tempting to speculate that the identified 6K1 protein is the predominant product of proteolytic processing. However, precursors present in infected tissue could have been lost during the purification process or not been recognized by the antiserum, as shown for TEV 6K2 polyproteins (Restrepo-Hartwig & Carrington, 1994). Additionally, even though the recombinant virus was designed to preserve the conserved heptapeptide sequences mediating N1a cleavage, the possibility that cleavage kinetics were influenced slightly by the insertion of the histidine tag cannot be excluded. The cleavage rate of N1a also seems to be affected – at least to a certain degree – by the surrounding sequence (García et al., 1992; Merits et al., 2002).

So far, 6K1 protein has been predicted for most potyviruses on the basis of conserved flanking N1a-cleavage sites and, in addition, sequence-comparison studies (Adams et al., 2005; Fanigliulo et al., 2003; Glasa et al., 2002; Kekarainen et al., 1999; Lain et al., 1989; Mouri et al., 2002; Riechmann et al., 1992; Sakai et al., 1997). Experimental evidence supporting the existence of mature 6K1 is mainly derived from cleavage studies of certain potyvirus species. Whereas efficient cleavage at site B (Fig. 2) is generally accepted, site A cleavage kinetics appear to be rather slow. Thus, partial cleavage at site A, between P3 and 6K1, was shown to occur for PPV in vitro (García et al., 1992) and for Potato virus A (PVA) in vivo by using a baculovirus–insect-cell system (Merits et al., 2002). PVA 6K1 was shown indirectly to be part of several intermediates of polyprotein processing, P3–6K1 having a particularly long half-life. Furthermore, two proteins corresponding to P3 and possibly P3–6K1 were detected in tobacco vein mottling virus (TVMV)-infected cells with antiserum raised against bacterially expressed TVMV P3–6K1 (Moreno et al., 1998; Rodríguez-Cerezo & Shaw, 1991). Whereas processing at site A was not required for PPV viability, it still affected symptom expression, suggesting that processing at site A plays a relevant role in the infection cycle of PPV (Riechmann et al., 1995).

Therefore, our study, together with these previous studies, indicates that 6K1 is being processed from the polyprotein of several potyviruses in vivo, but the efficiency of processing of site A and whether it is processed in all potyviral species in planta remain to be determined. Thus, TEV site A is not processed in vitro (Chu et al., 1997; Parks et al., 1992), suggesting that mature 6K1 of TEV does not exist and might function exclusively within a P3–6K1 polyprotein (Riechmann et al., 1995). But failure to detect in vitro cleavage of TEV site A could also be due to suboptimal cleavage conditions within the in vitro system – as suggested by contradicting results from in vitro and in vivo cleavage studies on TVMV site A (Rodríguez-Cerezo & Shaw, 1991; Yoon et al., 2000). Consequently, TEV site A might eventually be processed in vivo, too.

Even though 6K1 is probably produced not only during PPV infection, but also during most potyviral infections, knowledge on possible functions of mature 6K1 protein is still scarce, as functional studies have mainly focused on the P3–6K1 genomic region (Chu et al., 1997; Dallot et al., 2001; Hjulsager et al., 2002; Johansen et al., 2001; Moreno et al., 1998; Sænæs et al., 2000). The few studies solely analysing 6K1 suggest replicative functions. The deletion of 6K1 from the PVA genome (which also abolished the processing of the resulting recombinant P3–CI junction), as well as several insertions within the N terminus of 6K1, rendered the resulting PVA mutants non-infectious (Kekarainen et al., 2002; Merits et al., 2002). The raised anti-6K1 antibody and the use of engineered pPPV-H6K1-NAT infectious clone will contribute to further elucidation of the role that the mature 6K1 protein plays within the viral infection cycle.

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