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Non-structural plum pox potyvirus proteins detected by immunogold labelling

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Abstract

Plum pox potyvirus (PPV) induces in infected *Nicotiana clevelandii* cells characteristic crystalline inclusions known as nuclear inclusions (NI) when located in the nucleus and as dense material (Dm) when located in the cytoplasm. Crystalline inclusions contain protease (NIa) and RNA-dependent RNA polymerase (NIb) proteins. It is now well established for all potyviruses that cylindrical inclusions contain CI helicase ATPase protein (Martin et al., 1992). The intracellular location of other non-structural PPV proteins remains unknown. Using *Escherichia coli* expression vectors, specific antibodies were obtained against P1, P3, 6K2 and NIb PPV proteins for which antibodies were not yet available. As expected, NIb antiserum labelled crystalline inclusions. P1, P3 and 6K2 proteins were present in both types of crystalline inclusions found in the nucleus and in the cytoplasm of PPV-infected leaves of *N. clevelandii*, suggesting that nuclear inclusions and dense material were composed of the same proteins. This composition is discussed.

Potyviruses form a group of long flexuous particles composed of a single-stranded RNA (ssRNA) about 10 Kb long encapsidated by about 2000 copies of capsid protein (CP). The RNA genome is expressed as a 350 kDa polyprotein precursor which is proteolytically processed into seven proteins denoted as P1, helper component (HC), P3, cylindrical inclusion (CI), protease (NIa), RNA-dependent RNA polymerase (NIb), CP as well as two putative small polypeptides 6K1 and 6K2, located on both sides of the CI protein. The processing and function of some of these proteins are still controversial, but most of them are thought to be multifunctional (Riechmann et al., 1992; Riechmann et al., 1995; Verchot and Carrington, 1995; Mahajan et al., 1996).

In PPV-infected cells of *Nicotiana clevelandii*, a previous immunocytochemical study has revealed the subcellular location of two of these proteins, CI and NIa (Martin et al., 1992). The 68 kDa CI ATPase-helicase protein is restricted to the cylindrical inclusions, whether they are pinwheels or laminated aggregates. The PPV NIa antiserum reacts with the nuclear inclusions (NI) and with the cytoplasmic dense material (Dm). In this paper we report the characterization of polyclonal antibodies against P1, P3, 6K2 and NIb proteins and the localization of these proteins in the cellular inclusions induced upon PPV infection.

To obtain the antibodies, recombinant DNA constructs, representing the P1, P3, 6K2 and NIb coding regions of PPV RNA, from 147 to 1070 nt, 2444 to 3494 nt, 5555 to 5714 nt and 7022 to 8576 nt respectively, were generated using synthetic oligonucleotides as primer for the PCR whose template was the full-length cDNA of PPV (Riechmann et al., 1990). Each amplified product was cloned in the expression vector peX according to the recommendations of Landerdiagnostic, New England, Biolabs.

To prepare the antisera, over-expressed proteins were purified after electrophoresis on 10% SDS-polyacrylamide gels (Laemmli, 1970). Following staining with Coomassie brilliant blue, the bands corresponding to the fused proteins were excised,

homogenized and subcutaneously injected to New Zealand white rabbits. Additional injections were given until the sera reached adequate titres measured by ELISA test.

In order to make antibody identification easier, PCR amplified products were also cloned in the pMAL-c2 vector (from Landerdiagnostic) which carried the maltose binding protein (MBP) that was serologically different from the β-galactosidase fused protein carried by the peX vector. This arrangement enabled us to characterise the antibodies specifically targeted on the viral proteins by eliminating the non-specific reactions against β-galactosidase. With the control pMAL-c2 plasmid, a protein with an apparent molecular weight of 52 kDa appeared on the SDS-PAGE, which corresponds to the MBP (Figure 1A). The four recombinant proteins, MBP-P1, MBP-P3, MBP-6K2 and MBP-NIb migrated on the same SDS-PAGE gel showing their predicted molecular weights at 76 kDa, 80 kDa, 50 kDa and 98 kDa, respectively (Figure 1A). Very large amounts of MBP-P1, MBP-6K2 and MBP-NIb were synthesised in *E. coli*, whereas cells containing the pMAL-P3 recombinant resulted in a smaller amount of this protein, suggesting that the expression of this polypeptide was toxic to bacteria.

Antisera were tested by immunoblotting of bacterial lysates harbouring the pMAL-c2 and the four recombinant plasmids. The proteins from the SDS-PAGE gels were transferred onto a nitrocellulose membrane using a liquid Trans-Blot apparatus (Bio-Rad). After blocking non-specific binding sites using non-fat milk, the membranes were incubated with PPV-specific antibodies diluted to 1/50 for P1, P3, 6K2 and NIb, then with biotinylated anti-rabbit IgG antibodies, followed by detection using 4-chloronaphtol (Martin et al., 1995).

The antiserum prepared against protein P1 reacted specifically with the 76 kDa fusion protein. It also reacted with a smaller protein also present in the pMAL-c2 bacterial extract (Figure 1B). Antisera prepared against proteins P3 and NIb reacted with an 80 kDa and 98 kDa protein, respectively and did not react with pMAL-c2 control (Figure 1B). The antiserum prepared against polypeptide 6K2 did not give a clear western blot reaction (data not shown). The 6K2 serum, like the other sera, gave, however, a positive ELISA response indicating a successful rabbit immunization.

The primary leaves of *N. clevelandii* were inoculated with PPV Rankovic isolate and young systemically-infected leaves obtained 10 days after inoculation were used for electron microscopy experiments. Small

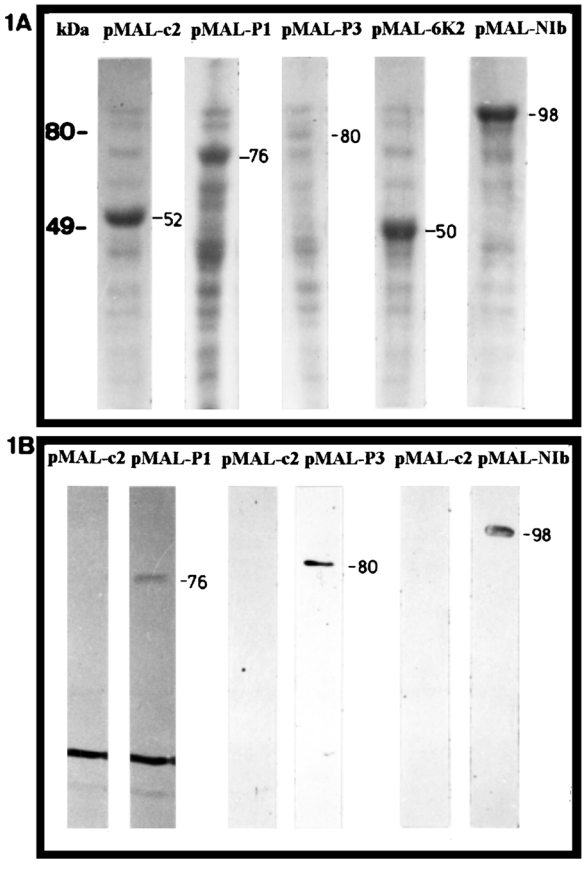
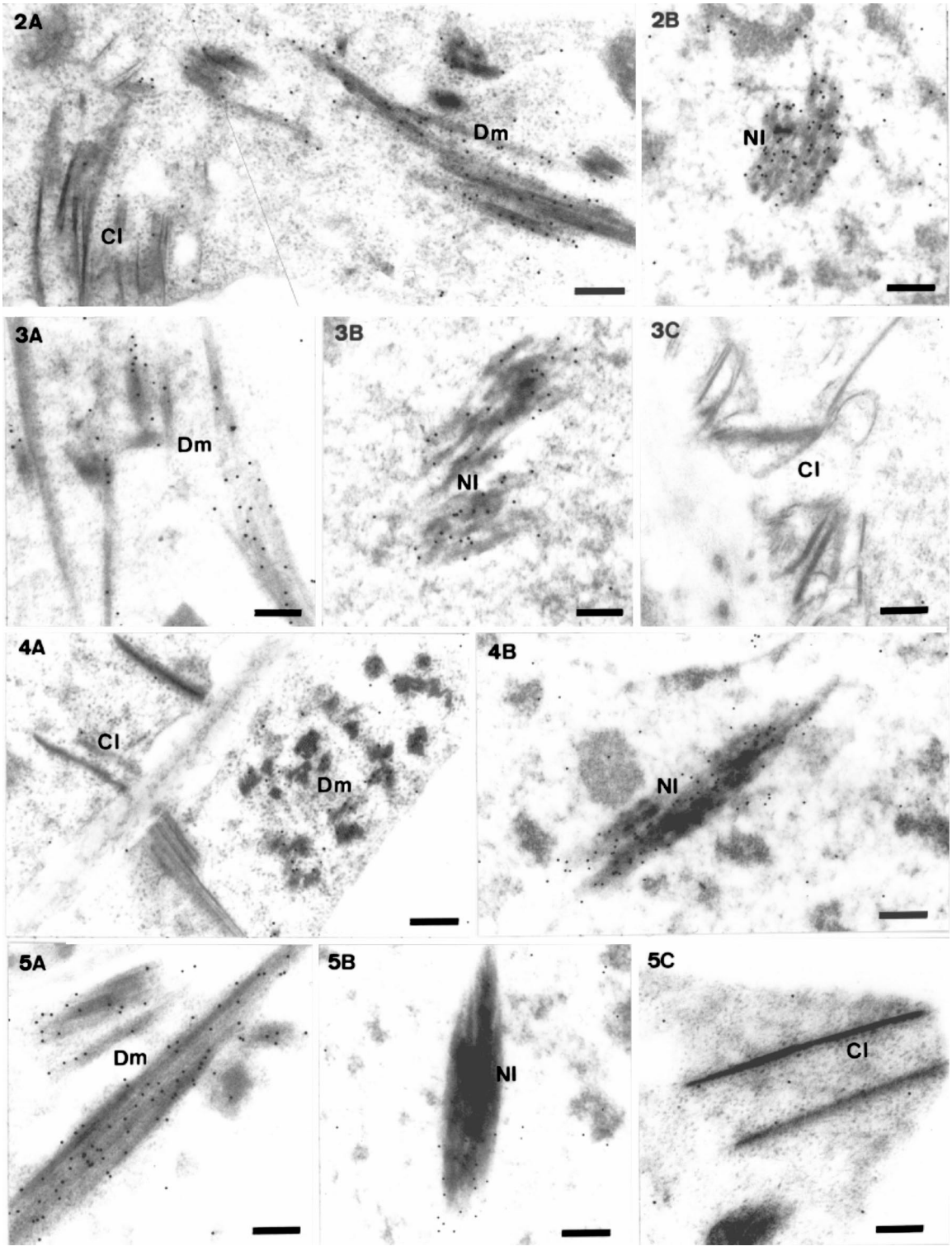


Figure 1. SDS-PAGE analysis of the expression of the pMAL-PPV-fusion proteins whose positions are indicated at the right of each lane. (A) Coomassie blue staining of proteins from the transformed bacteria (5 μl) separated on 10% SDS-PAGE gel. (B) Immunoblot analysis of the proteins separated as in (A), blotted onto a nitrocellulose filtre and probed with respective antisera.

pieces of *N. clevelandii* leaf tissue were fixed, dehydrated and embedded in LR Gold (LRG) as described by Martin et al. (1992). Ultrathin sections of infected and healthy tissue were labelled with 1/100 dilutions of specific rabbit antibodies, followed by detection using protein A-gold complexes of 10 nm or 15 nm in diameter (obtained from Biocell). After staining with uranyl acetate, they were observed in a Philips EM 300 transmission electron microscope at 80 kV.

Immunogold labelling with PPV NIb antiserum demonstrated a specific association with the dense material (Dm) located in the cytoplasm (Figure 2A) and with the nuclear inclusions (NI) located in the nucleus (Figure 2B) of infected *N. clevelandii* cells. No other virus-induced structures, in particular the cylindrical inclusions (CI), showed evidence of NIb labelling above the background (Figure 2A). This



Figures 2–5. Ultrathin sections of PPV-infected *N. clevelandii* cells incubated with the antisera against NIb, 6K2, P3 and P1, respectively. Figures 2A, 3A, 4A and 5A: Gold labelling is present on cytoplasmic dense material (Dm). Figs 2B, 3B, 4B and 5B: Nuclear inclusions (NI) are specifically labelled. Figures 2A, 3C, 4A and 5C: Cylindrical inclusions (CI) remain unlabelled. Bars represent 0,25μm.

result is similar to our previous findings namely that an antiserum to PPV-NIa also specifically labels the dense material and nuclear inclusions (Martin et al., 1992). Similar crystalline nuclear inclusions composed of equimolar amounts of NIa and NIb, which possess nuclear transport signals (Restrepo et al., 1990; Carrington et al., 1991) are induced by tobacco etch virus (TEV) (Dougherty and Hiebert, 1980). The existence of an independent nuclear translocation signal of TEV NIb, which directs efficient nuclear transport of a β-glucuronidase-NIb (GUS/NIb) fusion protein, has been reported in transformed *Nicotiana tabacum* (Li and Carrington, 1993). The TEV NIa protein was also known to be translocated to the nucleus (Restrepo-Hartwig and Carrington, 1992) when expressed *in planta*. However, PPV NIa and NIb seem to have a different behaviour, since they were found in both cell compartments.

Figures 3A, 3B and 3C show the immunocytochemical reactions of the virus-induced structures when the 6K2 antiserum is applied. The 6K2 polypeptide was specifically associated with the dense material in the cytoplasm (Figure 3A) and with the nuclear inclusions detected in the nucleus (Figure 3B). No labelling was found either on or in the neighbourhood of cylindrical inclusions (CI) (Figure 3C). The 6K2 serum gave specific labelling on crystalline inclusions as clearly as the other sera did, although it did not clearly recognize 6K2-protein epitopes under SDS denaturing conditions. The 6kDa protein from TEV (the equivalent of PPV-6K2), in the context of a 6kDa/NIa oligoprotein, impedes nuclear translocation of NIa and 6kDa/GUS fusion protein and induces a membranous proliferation associated with the periphery of the nucleus, on *N. tabacum* plants (Restrepo-Hartwig and Carrington, 1994).

The reaction with PPV P3 antiserum demonstrated that protein P3 was almost exclusively associated with the dense material (Figure 4A) and the nuclear inclusions (Figure 4B), whereas the neighbouring cylindrical inclusions (CI) were not labelled (Figure 4A). In earlier stages of infection, the TVMV P3 protein was shown to be associated with the cylindrical inclusions in infected *N. tabacum* (Rodriguez-Cerezo et al., 1993). The disparate results obtained from the subcellular localization of potyviridae proteins make it difficult to hypothesise a single mechanism for the function of each of these proteins. A prevailing mechanism may exist with some variations depending on virus-plant interactions.

Finally, the immunogold subcellular localization of protein P1 was shown for the first time. The reaction with PPV P1 antiserum demonstrated that protein P1 was specifically associated with the dense material (Figure 5A) and the nuclear inclusions (Figure 5B), whereas the cylindrical inclusions (CI) were not labelled (Figure 5C).

In all cases, the specificity of labelling was controlled on sections of uninfected tissue and on sections of infected cells incubated with preimmune serum and with anti-β-galactosidase sera. These controls showed no labelling above the background. No gold labelling was observed in either the dense material or the nuclear inclusions.

Our data suggest that the dense material and the nuclear inclusions are composed of the same proteins. It is conceivable that partially non-processed polyproteins, including P1-HC-P3 and 6K2-NIa-NIb, took part in such crystalline inclusions. Immunoblots conducted on protein extracts of PPV-infected plant tissues could further show whether processing of the polyprotein is complete or not.

As all virus-encoded proteins were initially expressed as a large polyprotein, the crystalline inclusions may represent an inactive accumulation of excess proteins or partially non-processed polyproteins that are able to cristallize. It appears unlikely that at these sites of over-produced crystallized gene products, the proteins are functional for the viral replication which occurs in membrane-bound complexes, as it is widely accepted (Martin and Garcia, 1991). Such an idea is supported by the fact that PPV glycerol gradient fractions with RNA polymerase activity contain at least NIa, NIb and CI proteins and cosediment with the endoplasmic reticulum membranes (Martin et al., 1995).

It is clear that further studies are necessary to conclusively demonstrate the role of PPV proteins in virus replication, movement and host cell interactions. Possible clues could be obtained from time course studies of infected plant tissues, associated with *in situ* hybridization of viral RNA.

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