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Ultrastructural localization of nonstructural and coat proteins of 19 potyviruses using antisera to bacterially expressed proteins of plum pox potyvirus

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Summary. Antisera to the bacterially expressed nonstructural proteins (NSP) HC-Pro, CI, NIa, and NIb and the coat protein (CP) of plum pox potyvirus (PPV) were used for analysing the composition of virus-induced cytoplasmic and nuclear inclusions by electron microscopy. The antisera reacted with NSP and CP of PPV on immunogold-labelled ultrathin sections. Antiserum to CP reacted with virions of seven out of 18 other potyviruses. CP was distributed throughout the cytoplasm of infected cells. Antisera to PPV NSP specifically reacted with virus-specific cytoplasmic and/or nuclear inclusions induced by 17 different potyviruses. NSP were furthermore localized in confined cytoplasmic areas in between complex accumulations of virus-specific inclusions. Cylindrical inclusions induced by the potyviruses were proven to consist of CI protein. Most other cytoplasmic or nuclear inclusions were shown to be composed of two or more NSP. An unexpected composition of virus-induced inclusions was observed for the crystalline nuclear inclusions of tobacco etch virus. Here, in addition to the expected presence of NIa and NIb, HC-Pro could be demonstrated. Furthermore, amorphous cytoplasmic inclusions induced by papaya ringspot virus contained the expected HC-Pro but additionally NIa, NIb and CI. Beet mosaic virus-induced nuclear inclusions ('satellite bodies') contained in their electron-dense matrix NIa, NIb, Hc-Pro and CI and in their lacunae CP in bundles of virion-like filaments. The results indicate that all cytoplasmic or nuclear inclusions of potyviruses have to be regarded as deposition sites of excessively produced viral NSP.

Introduction

Potyviruses induce well known morphologically distinct cytoplasmic and/or nuclear inclusions [17, 19, 32], whose constituents, however, have been analysed only in a few cases [16, 25, 38]. Each potyvirus induces cylindrical inclusions commonly known as 'pinwheels' (PW), which are reported to consist exclusively of cylindrical inclusion protein (CI) [14, 24]. Cytoplasmic amorphous inclusions (AI) are morphologically less clearly defined than PW and are induced by a few potyviruses only. They appear as irregularly shaped, mostly rounded masses possessing a homogenous substructure of either finely granular material or tubular, fibrillar or rod-like elements [32]. Crystalline cytoplasmic or nuclear inclusions (NI) are more or less regularly shaped and the outer shape and fine structure of such inclusions is specific for the particular potyvirus. Crystals can be of rhomboidal (bean yellow mosaic- [BYMV], unidentified celery-poty [Poty-R], clover yellow vein- [C1YVV] viruses),truncated pyramidal (tobacco etch virus [TEV]) or elongated, needle-like shape (plum pox virus [PPV]) [32]. Crystalline inclusions can be restricted to the nucleus as with TEV, or the same crystal type can occur in both the nucleus and cytoplasm (PPV, BYMV, C1YVV, Poty-R), or the crystalline inclusions in the nucleus can differ from those in the cytoplasm (henbane mosaic virus [HMV]). Amorphous NI contain more or less condensed granular material (endive necrotic mosaic-[ENMV], eggplant severe mosaic-[ESMV], beet mosaic-[BtMV] viruses) or bundles of virion-like filaments (celery mosaic-[CeMV], chilli veinal mottle- [CVMV]). The electron-dense amorphous NI in BtMV-infected cells have been designated 'satellite bodies' [49], those induced by ENMV and ESMV [32] are similarly shaped, but have a less condensed matrix. It is generally accepted that all potyvirus-induced inclusions contain nonstructural proteins (NSP) that are functionally important for virus replication, cell-to-cell movement or vector transmission [52].

Seven major proteins are autoproteolytically processed from the polyprotein of potyviruses. Their designations and putative functions are: P1, protease; HC-Pro, helper component protein for aphid transmission and protease; P3, unknown function; CI, cylindrical inclusion protein, helicase and ATPase; NIa, nuclear inclusion protein A, protease and VPg; NIb, nuclear inclusion protein B, RNA-dependent RNA polymerase; and CP, coat protein [52].

Former evidence for the composition of potyvirus-induced inclusions has been based on Western-blot analysis of purified inclusions [12, 13, 23, 28, 35, 43]. However, direct information on the constituents of potyvirus-induced inclusions can be acquired by electron microscopy of ultrathin sections after immuno-gold labelling (IGL). Following bacterial expression of NSP, antisera to individual NSP have been produced and used to analyse virus-induced inclusions by immunoelectron microscopy [38, 40, 48].

In the present study, antisera to four different bacterially expressed NSP as well as CP of plum pox virus (PPV) were used for localizing the respective proteins in cells infected by PPV or 18 other potyviruses. Possible sites of viral replication in inclusions or cytoplasmic areas of potyvirus-infected cells are discussed.

Materials and methods

Protein expression

Using appropriate restriction enzymes and PCR primers, the genes coding for the seven major proteins of PPV-NAT were excised or amplified, respectively, from an infectious full length clone [36] and subcloned into the bacterial expression vectors of the pQE series (QIAexpress System, Qiagene GmbH, Hilden, Germany) or into the expression vector pTH [30]. Proteins were expressed and purified on Ni-NTA columns by affinity chromatography following the manufacturer's instruction [10, 26].

Antisera

Purified expressed proteins of PPV were used for immunising rabbits, and the resulting antisera were tested for specificity in DAS-ELISA [9] and IGL experiments. Since all antisera to bacterially expressed proteins reacted with healthy plant material, they had to be absorbed with a mixture of crude plant extracts from uninfected *Chenopodium quinoa* and *Nicotiana clevelandii* prior to their use for IGL experiments. For antiserum absorption, 1 g of leaf tissue of each plant species was ground in 1 ml of 0.1 M phosphate buffer (PB) pH 7.0, and centrifuged at 18 000 **g** for 5 min to remove plant debris. Four volumes of clarified extract were mixed with one volume of 1:10 diluted antiserum, incubated at room temperature for 1 h and centrifuged at 18 000 **g** for 5 min prior to use. Absorbed antisera to expressed proteins of PPV are referred to in the following tests as P1, HC-Pro, P3, CI, NIa, NIb and CP antisera. In addition, antisera to purified PPV, beet mosaic (BtMV) and endive necrotic mosaic (ENMV) virions (from the stock of the institute) as well as to purified CI of potato virus A (PVA; donated by H. J. Vetten, Braunschweig, Germany), to purified CI of potato virus Y and to HC-Pro of tobacco vein mottling virus (TVMV; both donated by T. P. Pirone, Lexington, Kentucky) were included in the experiments. As additional controls for all labelling reactions antisera to purified prunus necrotic ringspot virus (PNRSV) and the corresponding preimmune serum PNRSV0 (from the stock of the institute) were used.

Virus isolates

The 18 potyvirus isolates studied and their corresponding propagation hosts were as follows: CeMV, isolate 'Unna' in *Ammi majus*; Poty-R, isolate 'Radolfszell" in *Apium graveolens*; ENMV in *Chenopodium quinoa*; lettuce mosaic (LMV) in *C. amaranticolor*; Poty-N isolate from *Lagenaria* spp. in Niger and papaya ringspot (PRSV) both in *Cucurbita pepo*; BYMV, BtMV, bidens mottle (BiMoV), chilli veinal mottle (CVMV) and clover yellow vein (C1YVV), all in *Nicotiana benthamiana*; PPV, isolate 'NAT' in *N. clevelandii*; pepper veinal mottle (PVMV) in *N. glutinosa*; eggplant severe mosaic (ESMV) in *N. megalosiphon*; PVY in *N. tabacum* 'Samsun'; TEV and TVMV both in *N. tabacum* 'Samsun NN'; pepper mottle (PepMoV) in *N. tabacum* 'White Burley'; HMV isolate 'Atropa mild mosaic' in *N. tabacum* 'Xanthi'.

Tissue embedding for electron microscopy

Approximately 1 mm^2 samples from systemically infected leaf tissues were fixed either for 2 h in 2.5% glutaraldehyde in PB at room temperature or for 17 sec in 2% glutaraldehyde in PB in a microwave oven [6]. After dehydration in a graded series of ethanol (or acetone) (30% at 4 °C, 50%, 70%, 90% at −25 °C), samples were put in flat embedding moulds (Beem), embedded in LR Gold resin (London Resin Co. Ltd., Woking, UK) and polymerised at −25 °C[55]. Parallel samples were fixed at room temperature for 1 h in 2.5% glutaraldehyde in PB, followed by 0.5% OsO_4 in PB for 1 h and over night incubation in 1% aqueous uranyl

acetate in H_2O. After dehydration in a graded series of acetone, samples were embedded in Epon 812 resin (Serva) and polymerised at 60 °C [29].

Immuno-gold labelling

To detect viral proteins in infected plant cells, IGL experiments were carried out on ultrathin sections essentially as formerly described for LR Gold- [55] and Epon-embedded tissues [5]. Independently of the different reactivities of antisera in DAS ELISA, working conditions for IGL were optimized for maximal labelling intensity and minimal background by varying dilutions of individual antisera and GAR gold conjugates as well as the incubation times. Nonspecific binding sites were blocked for 15 min in PB containing 0.5% BSA. Ultrathin sections of LR Gold-embedded tissues were incubated for 2 h on 10–20 µl droplets of absorbed antiserum diluted 1:2500 in phosphate-buffered saline containing 0.05% Tween (PBS-T). Because of low reactivity, antiserum to CI was used at a dilution of 1:1000 in PBS-T. Incubated sections were subsequently washed in a stream of PBS-T, blotted briefly on filter paper and incubated for at least 2 h on 10–20 µl droplets of goat anti rabbit (GAR) IgG conjugated with 10 nm gold (BioCell, Cardiff, UK) diluted 1:100 in PBS-T, blotted Sections were subsequently washed in a stream of H_2O, stained for 15 min with 1% aqueous uranyl acetate, washed with H_2O and subsequently dried. Samples were examined in a Zeiss EM 10C electron microscope.

For IGL labelling of OsO_4-fixed Epon-embedded tissues, 100–120 nm thick sections were incubated for 30 min in 15% aqueous $NaIO_4$, washed with PB [5] and labelled essentially as described above, but extending the gold-conjugate treatment to overnight incubation. As controls for IGL labelling experiments, healthy plants embedded in parallel were labelled similarly.

Alternatively, silver-enhancement of GAR conjugated to 1 nm or 5 nm gold colloids was occasionally used following recommendations of the manufacturer (R-gent, Aurion).

IGL experiments also were done with crude extracts of infected plants [34]. Leaf tissue was homogenized in 0.1 M phosphate buffer pH 7.0. The crude exctracts were adsorbed for 5 min to pioloform-carbon coated grids, decorated by incubation with antisera at appropriate dilutions, viz. 1:200 for CP antiserum and 1:50 for NSP antisera, labelled with 10 nm gold-GAR conjugates as described above and negatively stained with 1% uranyl acetate.

Quantification of labelling

Specific gold labelling on ultrathin sections was recorded when the number of gold grains per area of a given structural element obtained with the given antiserum exceeded that on background label as observed on cell constituents like chloroplasts, mitochondria, cell walls and vacuoles. The label on such structures was similarly low in infected as well as uninfected cells (compare Table 3). Labelling intensity on a given structure was examined at a magnification of 8.000 to 20.000 × and graded into three classes:− = background (= random-labelling); + =labelling >2 × background; ++ = strong labelling >50 × background. However, a clear-cut assessment of gold particles per standard area could not be achieved for all labelled structures due to extremely irregular inclusion shapes and poorly defined inclusion contours.

Results

Antisera to bacterially expressed PPV proteins and their reactivity with plant extracts

PPV genes coding for the coat protein and nonstructural proteins (Fig. 1) were subcloned into appropriate expression vectors (Fig. 1) for protein expression in *E. coli*.

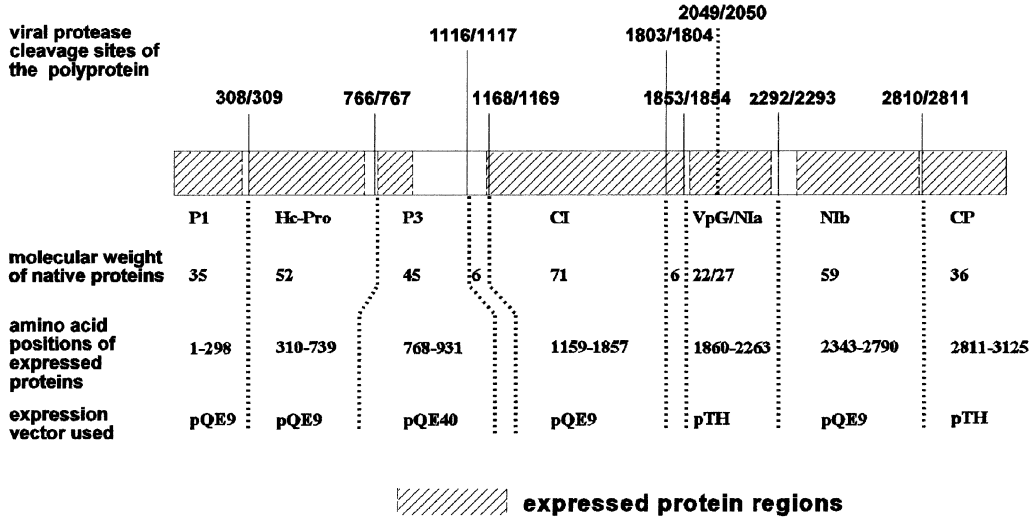

Fig. 1. Map of the PPV-polyprotein indicating positions of expressed proteins used for immunisation (hatched regions)

Table 1. DAS-ELISA reaction of the non-absorbed antisera to bacterially expressed PPV proteins with extracts from PPV-infected and noninfected *N. clevelandii* plants as well as with some expressed PPV proteins used as immunogens

	Antisera to						
	P1	HC-Pro	P3	CI	NIa	NIb	CP
extract infected plant[c]	0.054[a]	1.426	0.027	0.038	0.074	0.096	3.39
extract healthy plant[c]	0.003	0.002	0.017	0.004	0.012	0.016	0.3
expressed protein[d]	2.98	n.t.[b]	0.27	n.t.	n.t	1.83	n.t.

[a]DAS-ELISA readings (A_{405nm}) after substrate period of 20 h

[b]*n.t.* Not tested

[c]DAS-ELISA readings (A_{405nm}) with 20 mg homogenized plant material after substrate incubation period of 20 h

[d]DAS-ELISA readings (A_{405nm}) with 4μg/ml bacterially expressed homologous protein after substrate incubation period of 20 h

Antisera to the bacterially expressed PPV proteins varied considerably in the strength of their DAS-ELISA reactions with extracts from PPV-infected plants. In contrast to the strong reaction of the antisera to HC-Pro and CP, those to P1, CI, NIa, and NIb only gave weak but specific reactions. The P3 antiserum failed to react with PPV-infected plant extracts (Table 1). When some antisera were additionally tested against their homologous expressed proteins in DAS-ELISA, those to NIb and P1 gave strong reactions whereas P3 antiserum showed no specific reaction.

In IGL decoration tests [34] CP antiserum reacted strongly with PPV particles whereas NSP antisera failed to reveal reactions with PPV virions. Virions were

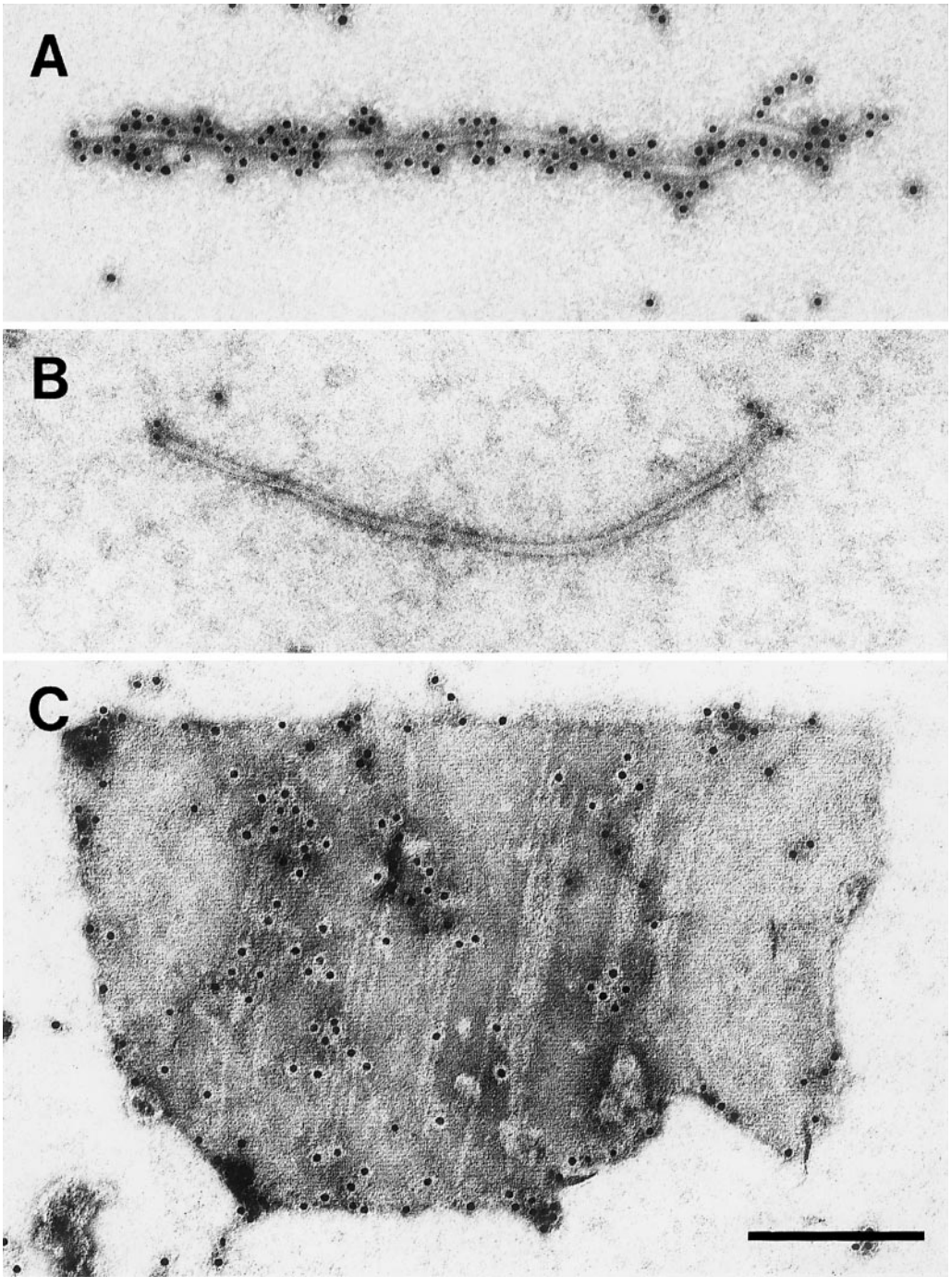

Fig. 2. Immunogold labelling of negatively stained virions or elements of cylindrical inclusions in plant extracts. **A** PPV virion with antiserum to PPV-CP, **B** ClYVV virion with antiserum to PPV-CP, **C** pinwheel-plate of PPV with antiserum to PPV-CI. Bar: 200 nm

relatively irregularly decorated by the CP antiserum (Fig. 2A) as compared to the uniform decoration obtained with an antiserum to PPV virions (not shown). Virions of seven other potyviruses viz. BtMV, BYMV, Poty-R, PepMoV, PRSV, TEV and TVMV, were not decorated nor gold-labelled by the PPV CP antiserum. However, ClYVV virions became gold labelled only at both ends (Fig. 2B). In heterologous labelling tests the antiserum to PPV virions detected TEV, Poty-R, and PRSV virions, whereas the PPV CP antiserum did not react.

Cylindrical inclusion elements occasionally found in crude extracts of PPV-infected leaves reacted only with antiserum to CI but not with those against CP and other NSP (Fig. 2C). Furtheron, in crude extracts the CI of the eight potyviruses studied were not labelled by PPV CI antiserum. None of these PW reacted with the antisera to other PPV-NSP although those of BYMV showed gold labelling on ultrathin sections by the same antisera (see below).

As described in Materials and methods the antisera could be used for specific labelling on ultrathin sections only after absorption with crude plant extracts. However, the absorption caused a decrease of specific reactivity by approximately two dilution steps. In decoration titer experiments with PPV particles, the crude and the absorbed CP antisera and the antiserum to PPV virions yielded titers of 1:3200, 1:1800 and 1:6400, respectively, indicating that the antisera to expressed CP and to PPV virions had principally comparable titers. Since the absorption was essential for a specific labelling on the sections, the loss of activity by the absorption as exemplified by decoration titer determinations had to be tolerated.

Localization of NSP and CP in cells infected by PPV

Significant IGL was obtained with tissues fixed in glutaraldehyde and embedded in LR Gold resin as well as with tissues fixed in glutaraldehyde and OsO_4 and embedded in Epon 812. Although a lower IGL intensity was obtained with Epon-embedded tissues, a difference in specificity was not evident. This permitted the use of Epon-embedded materials from previous studies, and results obtained with LR Gold-or Epon-embedded tissue were analysed without consideration of the embedding method.

The antisera to HC-Pro, CI, NIa, NIb and CP often yielded intensive IGL with ratios between specific label and background of more than 50:1 (Table 2). The specific labelling of cylindrical inclusions (Fig. 3A) with antiserum to CI, and of crystalline nuclear (Figs. 4A, C) and cytoplasmic (Figs. 4B, D) inclusions by antisera to both NIa and NIb confirmed the published localization of these NSP in PPV-infected cells [38]. In addition, the HC-Pro antiserum labelled AI found for the first time in PPV-infected cells (Fig. 3B). The CP antiserum detected virions in loose aggregates (Fig. 5A) as well as in more dense arrays (Fig. 5B) and furtheron in lomasome-like structures between the cell walls and the plasmalemma and, occasionally, also within plasmodesmata (Fig. 5C). Antisera to P1 and P3 yielded no labelling on sections. Because of the failure of the P1 and P3 antisera in IGL, they were not used in further experiments.

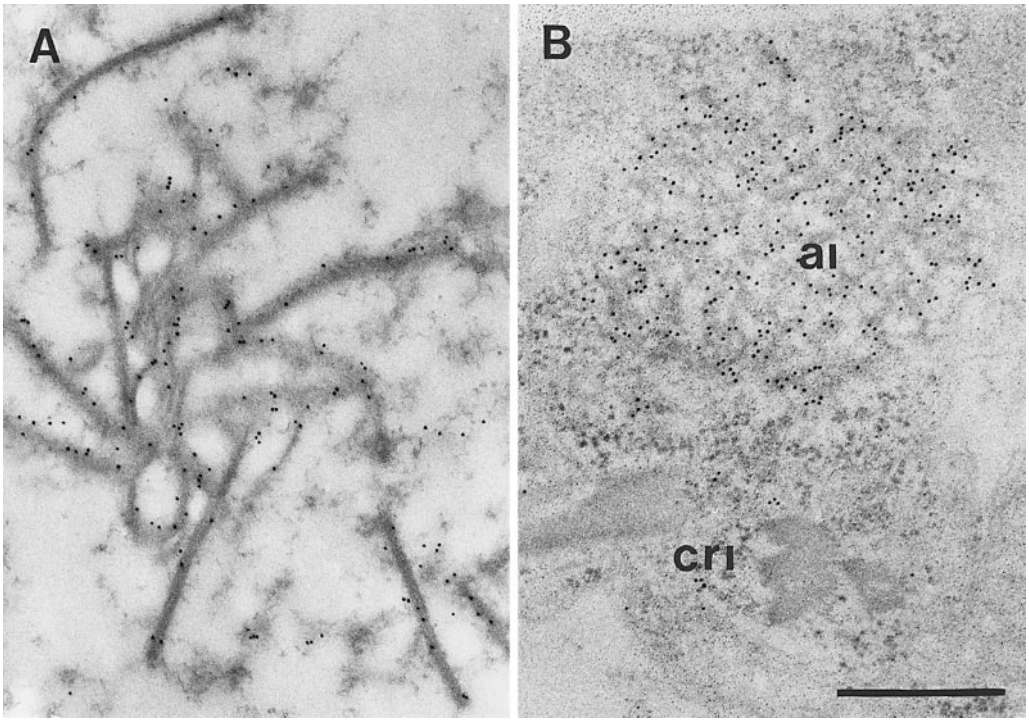

Fig. 3. PPV-induced structures in infected cells of *Nicotiana clevelandii*. **A** PW labelled with antiserum to PPV-CI. **B** Amorphous inclusion in the cytoplasm labelled with antiserum to PPV-Hc-Pro. Bar: 500 nm. Abbreviations used throughout the figures: *ai* amorphous inclusion in the cytoplasm, *ch* chloroplast, *cri* crystalline inclusion, *cw* cell wall, *lo* lomasome-like structure, *n* nucleus, *pw* pinwheel, *v* vesicle

Cross-reactivities of antisera or antigens could not be recognized. Labelling patterns of the antisera were always specific for definite structures. Antisera to the two NI proteins labelled the same inclusion structures of PPV. But differential reactions with inclusions of some heterologous potyviruses indicated their distinct labelling potential.

Localization of heterologous potyviral proteins of 18 potyviruses

The four NSP antisera and the CP antiserum possessed an unexpectedly broad heterologous IGL activity when tested on tissues infected by 18 distinct potyviruses. However, the five antisera differed considerably in their spectra of heterologous reactions. The NIa, NIb, HC-Pro and CP antisera each reacted with respective inclusions or virions of several of the viruses tested.

HC-Pro antiserum labelled the distinct AI of three potyviruses, viz. PRSV (Fig. 6A), PepMoV and TVMV (Table 2) irrespective of the fine structure of these inclusions. Those of PRSV (Fig. 6), like those of PPV (Fig. 3B) were typically granular AI, those of PepMoV were composed of tubular elements and those of TVMV of fibrillar elements. With three potyviruses (BiMoV, Poty-R, Poty-N) HC-Pro antiserum gave rise to scattered weak labelling signals in cytoplasms of

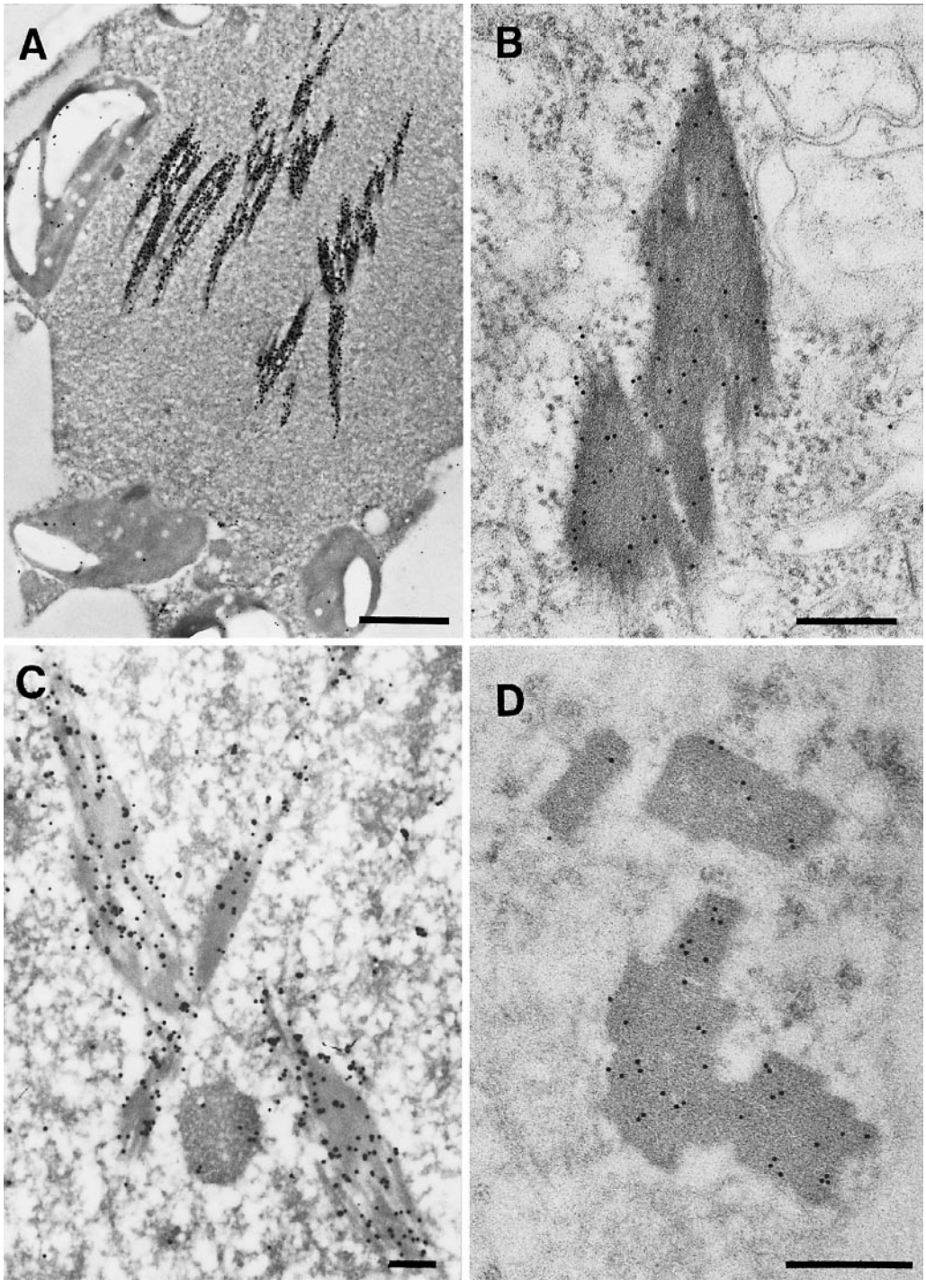

Fig. 4. Crystalline PPV-induced inclusions in infected *Nicotiana clevelandii* cells, **A** in the nucleus, detected with antiserum to PPV-NIa, silver-enhanced IgG gold conjugate, **B** in the cytoplasm detected with antiserum to PPV-NIa, **C** in the nucleus, detected with antiserum to PPV-NIb, silver-enhanced IgG gold conjugate, **D** in the cytoplasm detected with antiserum to PPV-NIb. **A** Bar: 2 μm; **B–D** Bar: 250 nm

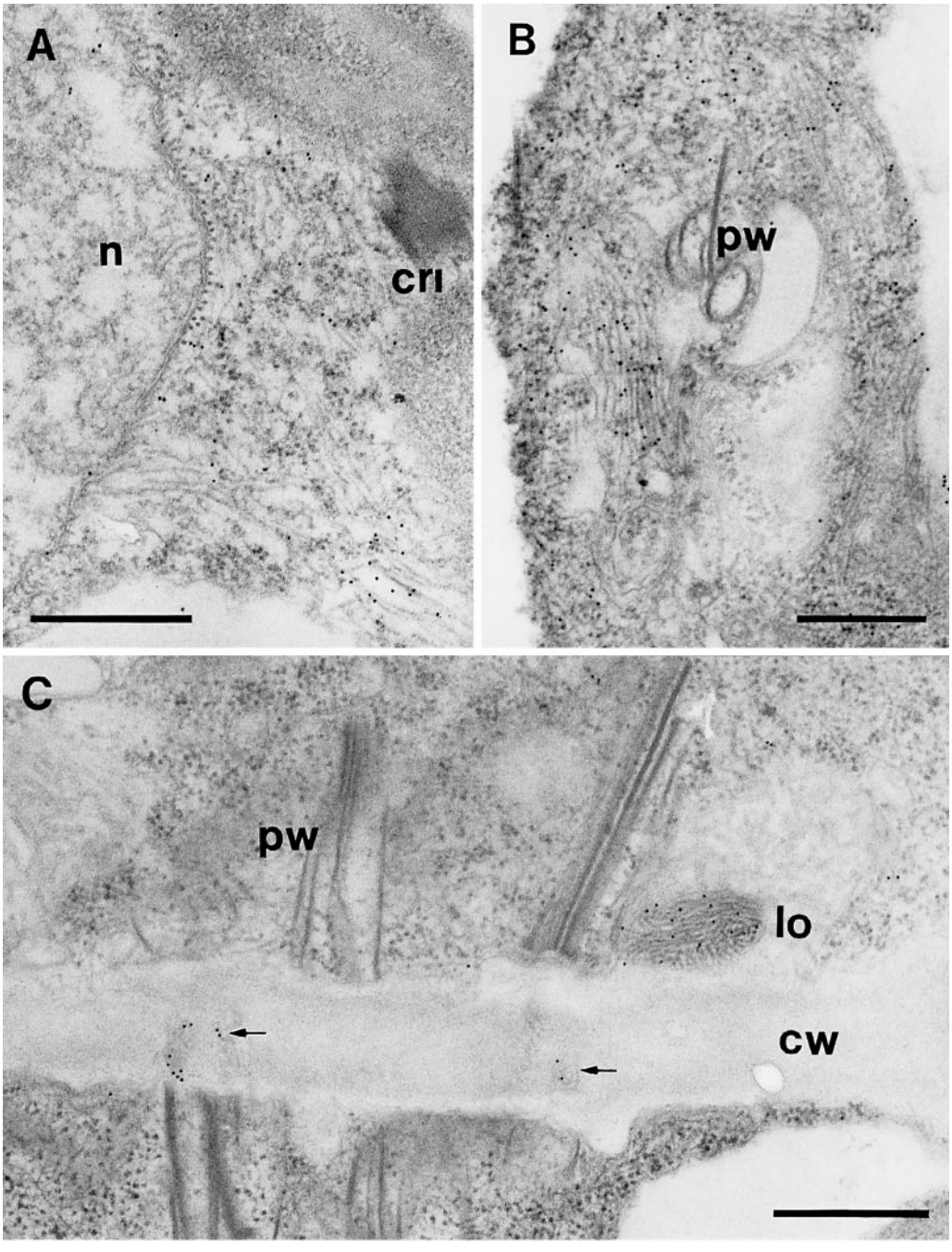

Fig. 5. Virions and virus-induced structures in PPV-infected cells of *Nicotiana clevelandii* labelled with antiserum PPV-CP. **A** Virions in scattered arrangement in the cytoplasm. **B** Virions in loose bundles in the cytoplasm. **C** Labelled filamentous material in a lomasome-like structure between the cell wall and the plasmalemma and in plasmodesmata (arrows). Bar: 500 nm

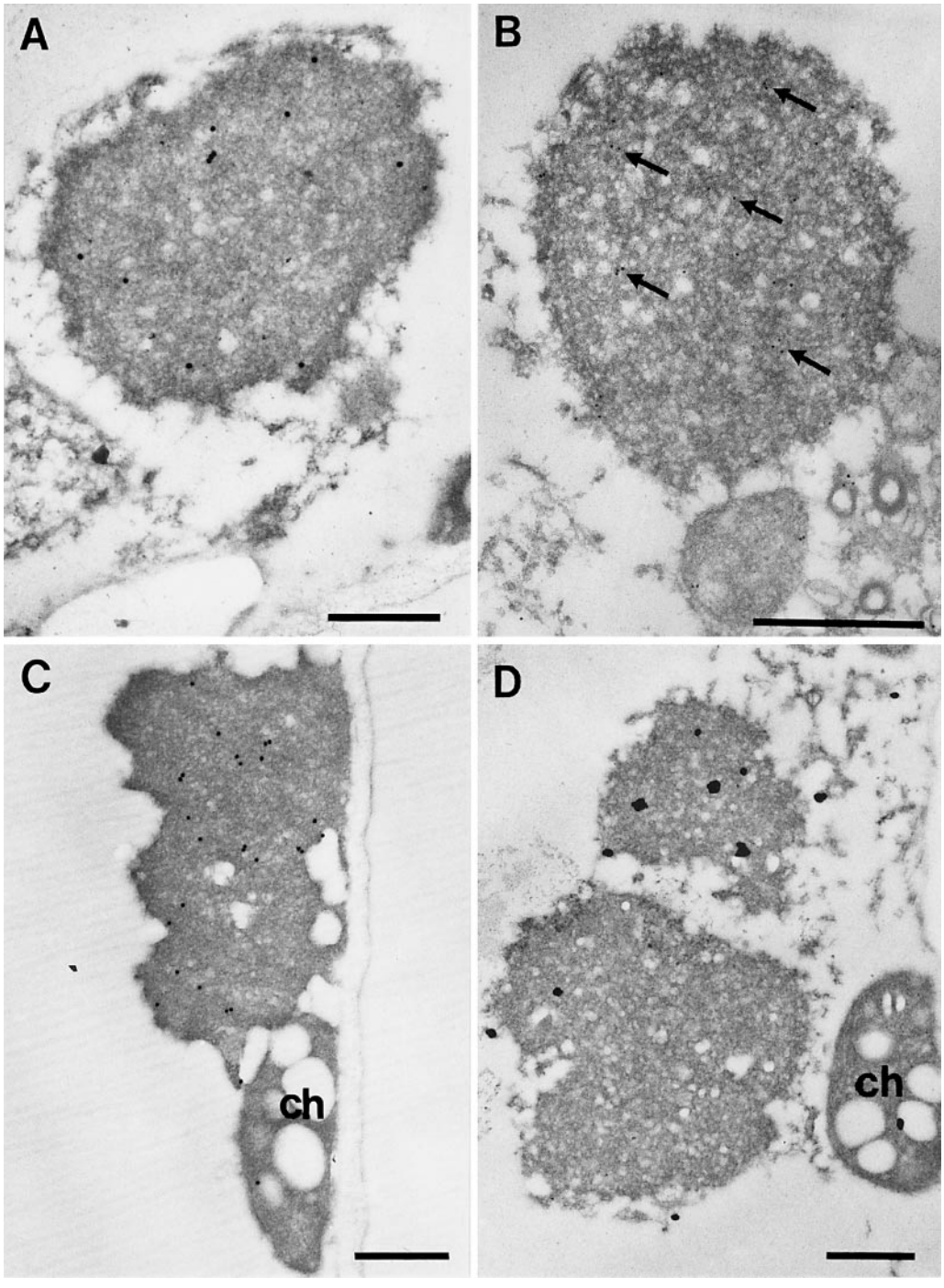

Fig. 6. Amorphous inclusions of PRSV-infected *Cucurbita pepo* labelled with antisera to recombinant PPV-NSP. **A** HcPro, **B** CI (arrows), **C** NIa and **D** NIb. Gold conjugates in **A, C** and **D** were silver-enhanced. Bar: 500 nm

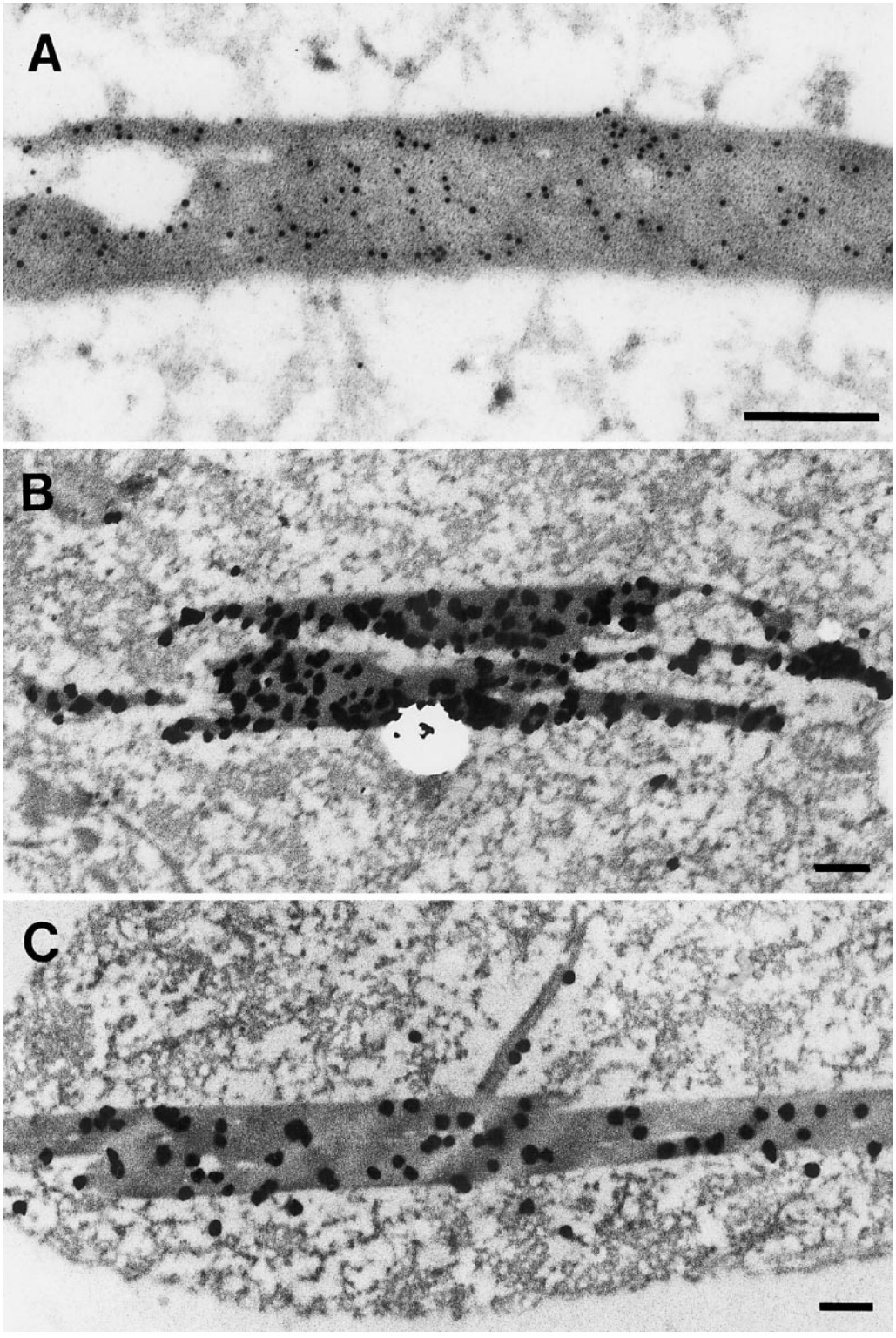

Fig. 7. Crystalline nuclear inclusion of TEV-infected *N. tabacum* 'Samsun NN' labelled with antisera to recombinant NSP of PPV. Label with **A** HcPro, **B** NIa and **C** NIb. Gold conjugates of **B** and **C** were silver-enhanced. Bar: 250 nm

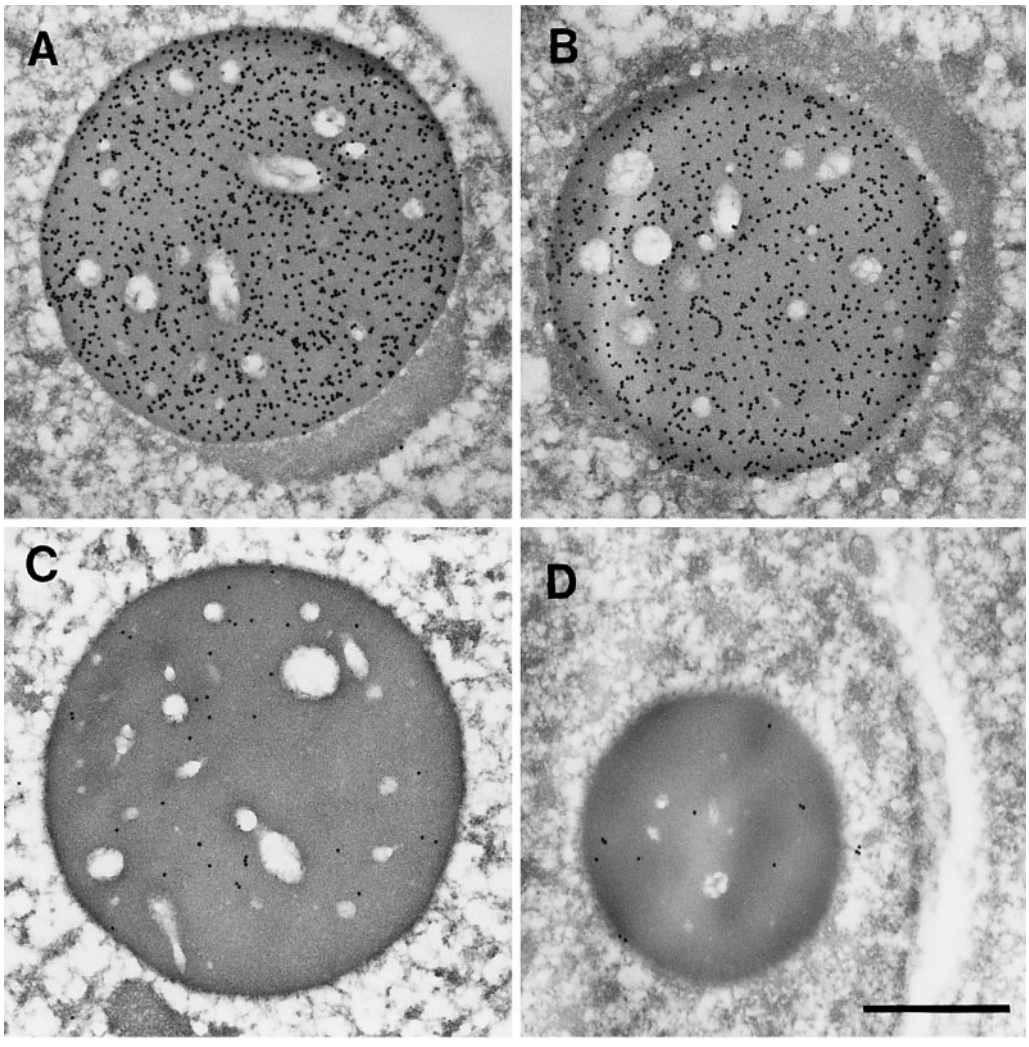

Fig. 8. Nuclear inclusion (*NI*)= 'satellite body', in nuclei of BtMV-infected *N. benthamiana* labelled with antisera to NSP of PPV. **A** NIa, **B** NIb, **C** HcPro and **D** CI. Bar: 1 μm

infected but not of noninfected cells. With BYMV and CIYV, the pinwheels were labelled by antiserum to HC-PRO. Furthermore, nuclear inclusions of TEV- (Fig. 7A) and BtMV-infected cells (Fig. 8C) were labelled at low intensity with HC-Pro antiserum. The HC-Pro antiserum did not react with cells infected by ENMV, ESMV, CVMV, HMV, LMV and PVMV (Table 2). In parallel tests with antiserum to TVMV HC-Pro, the AI of TVMV and PPV were labelled and additionally those of PVY (results not shown).

CI antiserum labelled the PW induced by BYMV, ClYVV, PepMoV, Poty-N, PRSV and TEV. Outside of PW, gold-labelling with CI antiserum was detected in nuclear inclusions of BtMV (Fig. 8D) and amorphous inclusions of PRSV (Fig. 6B). The PW of 11 potyviruses reacted neither with the CI antiserum nor with any other NSP antiserum. Further experiments (data not shown) with antisera to

Table 2. Identification of different viral proteins in potyvirus-infected cells by immuno-gold labelling with antisera against bacterially expressed proteins of PPV[a]

Virus[h]	Cylindrical inclusion (PW)-type	Detection only in cytoplasm	Cytoplasmic inclusions				Nuclear inclusion, amorphous or crystalline
			pinwheels (PW)	amorphous (AI)	crystalline	virus[f]	
PPV	III		CI[b] + +[d]	H++	A++ B++	CP++	A++ B++
BYMV	II		H++ CI+	/	B+	–	B+
C1YVV	II		H++ CI+	/	A++ B++	CP+	A++ B+
Poty-R	II	H+	–	/	A++ B+	CP++	A++ B+
HMV	I, IV		–	/	–	–	A+ B+
CVMV	IV		–	/	–	–	–
BtMV	II		–	/	/	CI+ A+ CP+[e]	H+ CI+ A++ B++ CP+[g]
ENMV	I, VI		–	/	/	CP+	A+ B++
TEV	II		CI+	/	/	CP++	H++ A++ B++
PVMV	IV		–	/	/	/	A+ B+
ESMV	I, IV		–	–	/	CP+	B+
TVMV	IV		–	H+	/	CP+	–
CeMV	I, IV		–	–	/	–	–
PRSV	I		CI+	H+ CI+ A+ B+	/	CP++	/
PepMoV	IV	A+	CI+	H+ B++	/	CP++	/
Poty-N	IV	H+	CI++	–	/	CP+	/
PVY	III, IV	A+ B+	–	–	/	CP+	/
LMV	II	B+	–	/	/	/	/
BiMoV	II	H+	–	/	/	/	/

[a]IGL was done as described in Materials and methods

[b]Abbreviations for the antisera to bacterially expressed proteins of PPV: *H* aphid transmission helper component protein; *CI* cylindrical inclusion protein; *A* nuclear inclusion protein a; *B* nuclear inclusion protein b; *CP* coat protein

purified PW of PVA and PVY were tested with tissues infected by the 19 viruses. Both antisera reacted with the PW of the five viruses which had also reacted with the antiserum to PPV CI. Additionally, antiserum to PVA PW reacted with those of BYMV, TVMV and PVY, and antiserum to PVY PW reacted with BYMV, ENMV, ESMV and PVY.

Antisera to both, NIa and NIb labelled the cytoplasmic crystalline inclusions induced by CIYVV and Poty-R as well as cytoplasmic amorphous inclusions of PRSV (Fig. 6C, D) (Table 2). Crystalline cytoplasmic inclusions of BYMV as well as cytoplasmic amorphous inclusions of PepMoV were labelled by NIb antiserum only. Cytoplasmic crystalline inclusions of HMV and CVMV were not labelled at all. Both antisera labelled the nuclear inclusions of 7 out of 12 NI-inducing heterologous potyviruses (Figs. 4A, 4C, 7B, 7C, 8A, 8B). The NI of BYMV and ESMV reacted only with NIb antiserum, while those of CVMV, CeMV and TVMV did not react with both antisera.

IGL obtained with CP antiserum was associated with scattered or aggregated virions in cells infected by 11 potyviruses. In contrast to NSP inclusions virions were not confined to certain cytoplasmic regions but were rather dispersed throughout the cytoplasm of infected cells. Virions of four viruses (CVMV, BYMV, HMV and CeMV) were not labelled whereas virions of PVMV, LMV and BiMoV could not be morphologically recognized on ultrathin sections of infected cells (Table 2). Furthermore, bundles of filaments present in the lacunae of 'satellite bodies' of BtMV [32, 49] were labelled with the CP antiserum (Figs. 9A, 9B). They were equally labelled by antisera to virions of BtMV, ENMV (Fig. 9C) and PPV, indicating that the filaments are in fact composed of BtMV-CP. In contrast, large bundles of tubular filaments in CeMV-infected nuclei did not react with NIa, NIb and CP antisera.

Detection of NSP outside distinct inclusions

IGL indicated presence of NSP in certain cytoplasmic regions apart from distinct inclusions. Weak label was not distributed throughout infected cells but

[c]Types of cylindrical inclusions according to Edwardson, Edwardson et al. [20], Edwardson and Christie [19]

[d]Labelling intensity: $++$ = strong labelling $> 50 \times$ background-labelling; $+$ = labelling $> 2 \times$ background; $-$ = random-labelling (= background); / = structure not present

[e]Observed structures appeared as dense and sharply confined aggregates of viruslike filaments which were not observed with the other potyviruses studied. Obviously they are not composed of virions only

[f]Virions appeared as more or less dense aggregates or as scattered particles in the cytoplasma

[g]CP labelling on bundles of virion-like particles in lacunae of nuclear 'satellite bodies'

[h]The potyviruses were grouped, and the table subdivided in order to illustrate the presence or absence of distinct inclusions containing NI-proteins in the cytoplasm and/or nuclei of infected cells (indicated by hatched areas)

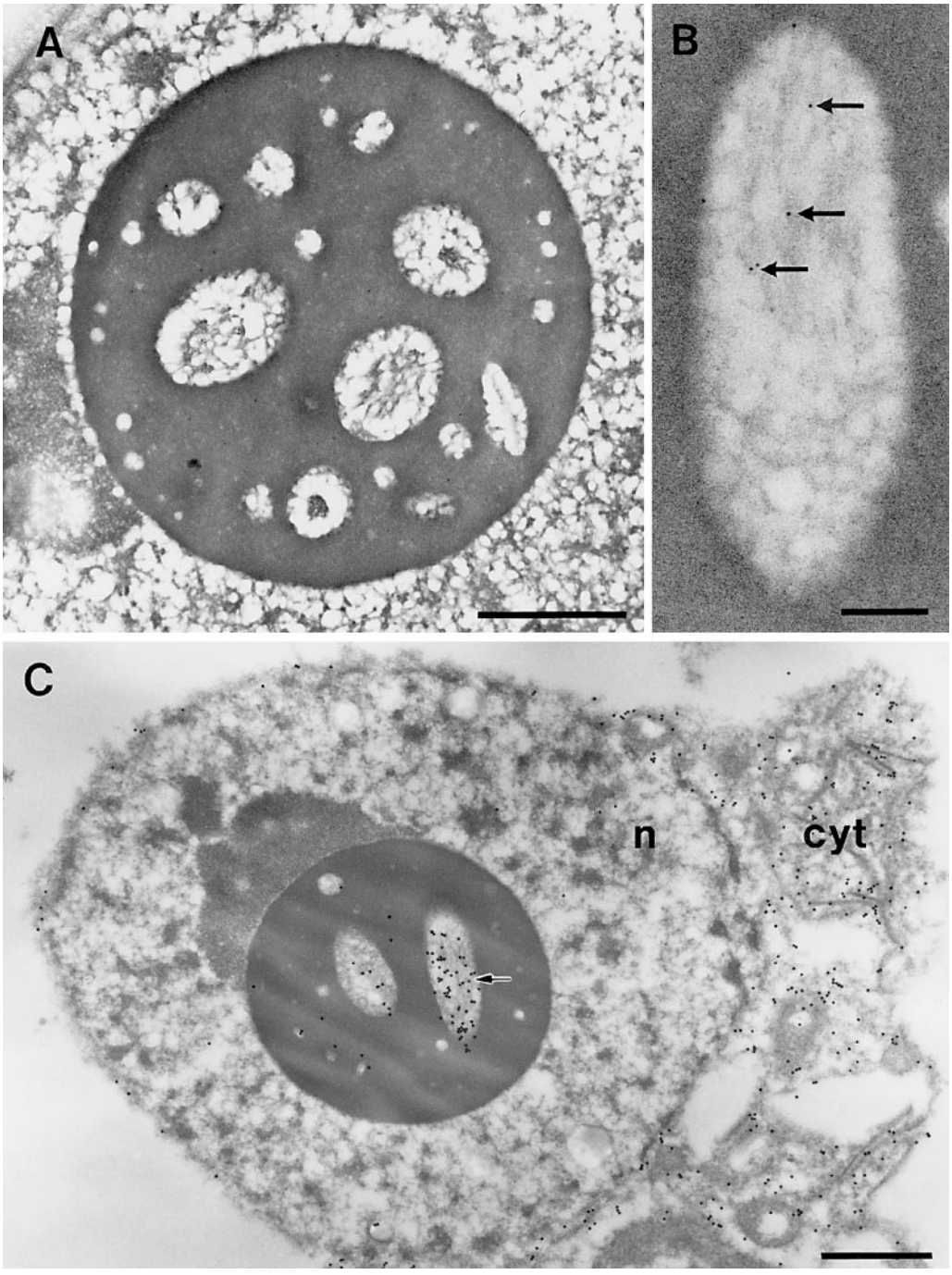

Fig. 9. Filamentous material in lacunae of BtMV-induced nuclear 'satellite bodies' labelled in **A, B** with antiserum to PPV-CP. **A** Overview, **B** one lacuna of a 'satellite body', **C** labelling with antiserum to purified virions of ENMV in the lacunae (arrow) as well as on virions in scattered arrangement in the cytoplasm (*cyt*). **A** and **C** Bar: 1 μm. **B** Bar: 250 nm

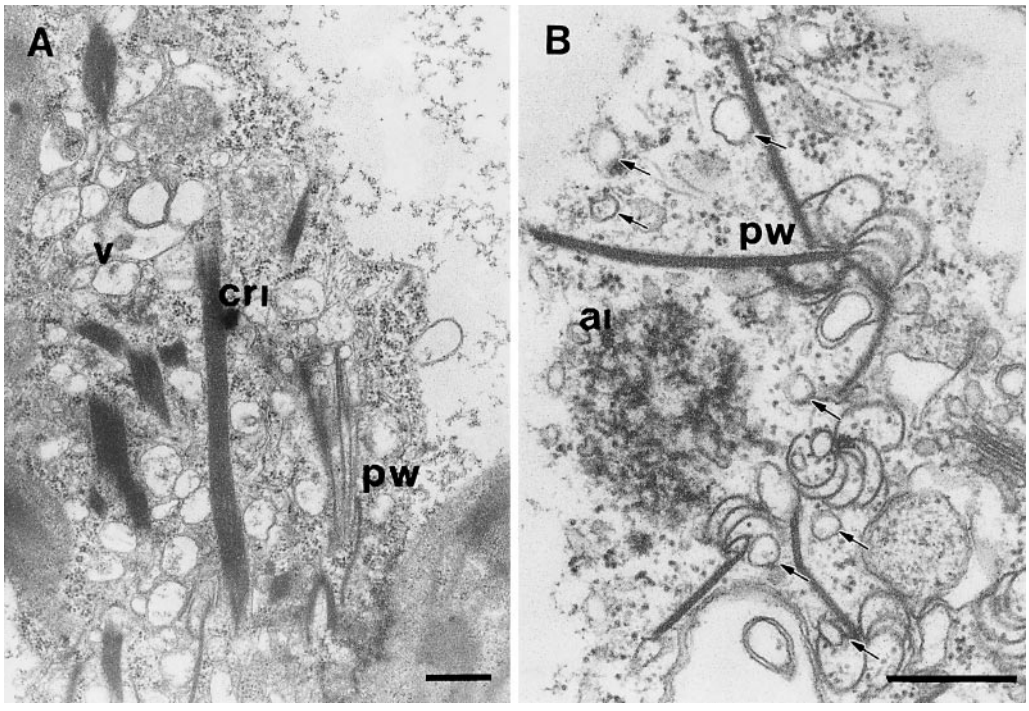

Fig. 10. PPV-induced structures in infected cells of *Nicotiana benthamiana* embedded in Epon 812. **A** Cytoplasmic vesicles between crystalline inclusions and longitudinally cut PW. **B** Vesicles in the cytoplasm near PW (arrows) and amorphous inclusion. Bar: 500 nm

appeared to be concentrated in cytoplasmic areas of the complex accumulations of inclusions and organelles induced by most potyviruses. In Epon-embedded tissue, such sites often contained clusters of small vesicles (Fig. 10). Corresponding membrane structures were not preserved in LR Gold resin, but the sites labelled by the used antisera corresponded to the areas containing vesicles in Epon. Since labelling density was rather low at such sites, the significance of the labelling was often difficult to assess, especially with the weakly reacting NSP antisera e.g. that to CI. Table 3 presents an evaluation of such labelling results on the basis of gold particle counts for the strong reacting NSP antisera to NIa, NIb and HC-Pro (Fig. 11). The areas chosen for particle counting were of necessity rather small, because only small areas of 'free' cytoplam could normally be recognized between the inclusions. Despite a high standard deviation of the gold particle counts, a comparison with control preparations proved that a significant reaction with these antisera was achieved.

Table 3. Identification of different nonstructural proteins of PPV in the cytoplasm of PPV-infected cells by IGL using NSP and control antisera[a]

Antiserum	HC-Pro[b]		NIa[b]		NIb[b]		RV3[c] (PNRSV)		pre-immune-serum[c]		NIb[b]
Evaluated cell region	incl+[d]	incl−[e]	incl+	incl−	incl+	incl−	incl+	incl−	incl+	incl−	non-infected plant[g]
Mean value[f]	13.27	0.75	2.35	0.4	2.3	0.4	0.3	0.2	0.4	0.3	0.15
Standard deviation	8.81	1.29	1.36	0.73	1.36	0.73	0.58	0.33	0.6	0.48	0.37

[a]The sections of LR Gold-embedded infected leaf tissues were incubated for 2 h on antisera diluted 1: 2500 in PBS-T, followed by incubation for 2 h with goat anti-rabbit gold conjugate diluted 1.50 in PBS-T and staining for 12 min in UAc

[b]Absorbed antisera to expressed proteins of PPV

[c]Nonabsorbed antisera to purified virions of prunus necrotic ring spot virus and the corresponding preimmune serum.

[d]'Free' cytoplasm in region of an infected cell, which contains virus-induced inclusions

[e]'Free' cytoplasm in region of an infected cell, which does not contain virus-induced inclusions

[f]Gold grains counted per 0,03μm^2 of 'free' cytoplasm

[g]Non-infected *N. clevelandii*

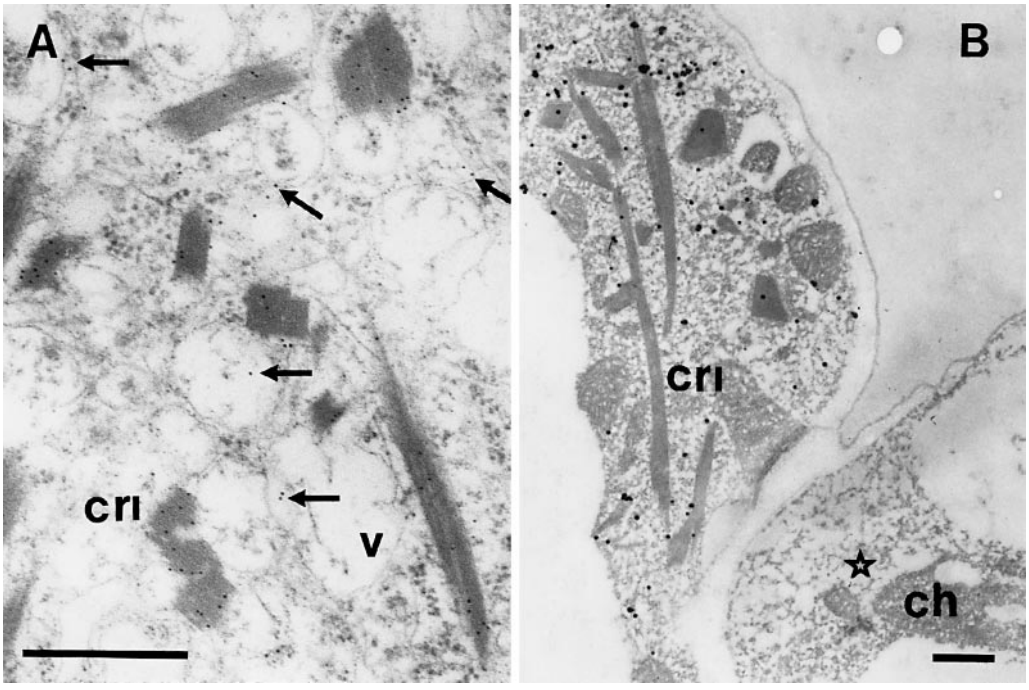

Fig. 11. NSP in the cytoplasm of PPV-infected *Nicotiana clevelandii* cells in distinct inclusions as well as between them. **A** Weak label with antiserum to PPV-NIb in the cytoplasm between distinct inclusions, sometimes associated with vesicles (arrows). **B** Label with antiserum to PPV-HcPro only in the cytoplasm between distinct inclusions, not in cytoplasm without these inclusions (asterisk). Bar: 500 nm

Discussion

Relative reactivity of antisera to bacterially expressed proteins in different test systems

In contrast to the broad reactivity of the antisera to NSP and the CP of PPV in IGL on ultrathin sections their relative reactivities varied considerably in different serological test systems (Tables 1 and 2). 1). The antisera to CP and HC-Pro both showed a strong reactivity in DAS-ELISA and in IGL. 2) The low ELISA reactivity of the antisera to CI, NIa and NIb combined with strong reactivity in IGL may be caused by low solubility of the respective NSP from their aggregates in extracts of infected cells. 3) The weak reactivity of the P1-antiserum in DAS-ELISA with native protein and in IGL, in contrast to strong reactions in DAS-ELISA with bacterially expressed P1 might indicate a high turnover of P1 in vivo or a conformation of native P1 which is not detectable by antibodies to bacterially expressed P1. 4) Attempts to express P3 from the whole sequence of its gene or from its 3′ part probably failed, because it was toxic to *E. coli*. Since the 5′ part could be successfully expressed but induced no serologically active antiserum detection of P3 failed completely.

Intracellular localization of NSP and CP of PPV

Our observations on proteins of PPV can be compared with published data on PPV and other potyviruses as follows. **P1**: antiserum to P1 of PPV did not yield IGL on ultrathin sections. The localization of P1 in cytoplasmic clusters described for ZYMV by Wisler et al. (1995) could not be confirmed. **HC-Pro:** the newly detected AI in PPV-infected cells had not been described till now [43]. In IGL with NSP-antisera, they were found to contain HC-Pro only. **P3:** the reported localization of TVMV-P3 at the cylindrical inclusions [47], respectively the association of PPV-P3 with the crystalline inclusions [39] could not be verified here. **CI:** CI-antiserum labelled the PW of PPV exclusively as described earlier [38]. **NIa and NIb:** these proteins were located together in both, nuclear and cytoplasmic crystalline inclusions similar as described by Martin et al. [38]. **CP:** The distribution of CP in wide areas of infected cells was similar to that described for TEV [2]. This distribution coincided with former cytopathological knowledge on PPV [18, 32, 54] and other potyviruses [31, 56]. Additionally, lomasome-like structures found here to contain CP resembled very much structures containing unidentified 'thread-like elements' which were described for BtMV [49]. By our labelling experiments we could not decide whether the filaments seen in PPV-induced lomasome-like structures or plasmodesmata are virions or other CP aggregations and whether they correlate to the mechanism of cell-to-cell transport.

Nonstructural proteins in virions and PW from suspensions?

No NSP could be shown to be included in the protein coat of the virions. Thus no indication was found for the presence of the genome-linked protein (VPg = N-terminal part of NIa; [27, 46]) at one of the particle ends [50]. Its detection with

the NIa antiserum could have been expected, since the expressed antigen used for the production of this antiserum in fact contained the whole NIa protein including its VPg part. However, we have no proof that this NIa antiserum reacts with VPg at all. Pinwheels suspended in plant extracts appeared to be composed of CI only. This was found also with PW of BYMV and ClYVV, although antiserum to HC-Pro labelled PW of these viruses in ultrathin sections. Obviously HcProc can be removed from these structures easily during tissue extraction, what indicates a relatively unstable association in vivo.

Localization of NSP and CP of heterologous potyviruses

The broad heterologous reactivity with sectioned virions in contrast to the very limited heterologous reactivity of CP antiserum with virions in plant extracts demonstrates that many epitopes detected by CP antiserum are cryptotopes made accessible to labelling only upon sectioning. Correspondingly, the relatively high intensity of heterologous IGL on NSP aggregates may result from multiple cryptotopes in aggregated NSP molecules made accessible on sections, but additionally also because within inclusions absolute concentrations of antigen are extremely high. The extensive heterologous reactivity of all antisera to NSP and CP of PPV allowed us to analyse the distribution of virus proteins in cells infected by a broad spectrum of potyviruses.

The results showed that similar to PPV, inclusions induced by most of the tested potyviruses contained one or more NSP. Examples for inclusions composed of one NSP are PW. They reacted only with CI antiserum or did not react with any antiserum to other NSP. Only with two of the potyviruses, BYMV and ClYVV, PW-associated label with HC-Pro-antiserum was observed. As discussed before HC-Pro appears not to be integrated in the PW structure.

As illustrated in Table 2, some of the analysed other inclusions were composed of three or more NSP (TEV, PRSV, BtMV). In these cases more NSP were proven to occur in the cystalline nuclear and amorphous cytoplasmic inclusions, respectively, than known before. The NI of TEV are not only aggregates of NIa and NIb as hitherto described [2, 13, 28] but unexpectedly contain also HC-Pro. In earlier Western-blot analyses of the composition of TEV NI an apparently single SDS-PAGE band in fact might have contained two proteins of nearly identical molecular weights like NIa with 49 kD (430 aa) and HC-Pro with nearly 51 kD (459 aa) [15, 52]. The included band of HC-Pro may have stayed unrecognized since unexpectedly antibodies to HC-Pro will also have been present in the used antisera to purified TEV-NI.

Cytoplasmic AI induced by PRSV and PepMoV are the classical inclusions in which the presence of HC-Pro had already been demonstrated [11, 12]. We confirmed this by heterologous IGL with PPV HC-Pro antiserum. However, we additionally detected NIa and NIb or only NIb, respectively, in PRSV and PepMoV induced AI. Thus it appears that also AI are more complex in composition than expected before [11, 12].

'Satellite bodies' of BtMV [49] have been analysed by us for the first time and were found to be a particularly complex example of multiple composed inclusions.

Since no former information on the composition of the 'satellite bodies' was available the significance of our results was corroborated by especially intensive control tests with other antisera.

It has been discussed previously whether the multiple composition of such inclusions might indicate an accumulation of incompletely processed polyprotein fragments [46]. This assumption may be difficult to prove with antisera to purified inclusions, since these might lead to overlooking of some components of inclusions if more than one NSP of nearly identical MWt are present (see TEV). The use of our antisera to expressed NSP could also not completely elucidate the occurrence of NSP in inclusions, since not all NSP were detectable for us. Indications for the presence of more NSP than we detected in PPV-induced crystalline inclusions were reported by Martin et al. [38] and Martin and Gélie [39], who additionally to NIb and NIa detected P3 and the NIa-associated 6K2 proteins in the same inclusions, respectively.

Serological relations between potyviruses on the NSP level

Heterologous serological relations between NSP detected by us (Table 2) correspond with most of the published, though scattered informations on the heterologous detection of components of inclusions induced by PPV, PRSV, PepMoV, TEV, TVMV, and PVY [3, 11, 12, 25, 28, 38]. Similarity of serological determinants of HC-Pro of PPV and TVMV, but dissimilarity of HC-Pro of PPV and PVY were shown by our results and had been reported on the basis of antisera to bacterially expressed PPV-HC-Pro [43] and to purified AI of PVY [3, 25]. De Meija et al. [11, 12] reported a difference between serological determinants of HC-Pro of PepMoV and PRSV. In contrast, our PPV-HC-Pro-antiserum reacted with the AI of both viruses. The proven serological relations of many of the studied potyviruses on NSP- and CP-level indicate the presence of common epitopes i.e. indicate conserved protein structures. However, with still further potyviruses the same antisera did not detect corresponding epitopes. In summary it could not be decided whether the common epitopes found for several potyviruses in our study correspond to serological relations known on the CP-level, since results obtained with IGL cannot adequately be differentiated by quantification.

Functional significance of inclusions

Our comprehensive results supplemented by earlier published work illustrate that no uniform pattern of distribution and composition of potyviral NSP aggregates exists. The heterogenous distribution in the infected cells, the diverse fine structures and composition of potyvirus induced inclusions as well as the lack of inclusion formation by several potyviruses suggest that the inclusions are in fact a heterogenous collection of NSP accumulations. Thus, no common cytological model for potyviral replication or protein synthesis in the inclusions can be deduced from our observations. Furthermore, reported site-specific properties e.g. in form of nuclear targeting sequences of TEV NI proteins [8, 44, 45] are obviously not generally specific for the particular NSP, since NI-protein-composed inclu-

sions can with other potyviruses be located in nuclei as well as in the cytoplasm (e.g. BYMV, ClYVV and PPV) or in the cytoplasm alone (PRSV, PepMoV). Thus a functionally specific significance of the nuclear targeting sequences in TEV-NI proteins appears unlikely. Also, the late appearance of inclusions in infected cells [4], after the bulk virus replication appears to be completed, supports the possibility that inclusions serve as pools of NSP as had been suggested by Baunoch et al. [4] for HcPro containing amorphous inclusions. The potyviral genome organization which comprizes a single open reading frame only prevents differential regulations of amounts of produced proteins. Thus some NSP are produced in amounts exceeding the functionally needed quantities, are accumulated in inclusions which, therefore, are merely sites of deposition.

Indications for the site of virus replication

Cylindrical inclusions are the only virus-induced inclusion produced by all members of the *Potyviridae* family and, therefore, might be supposed to be essential for the infection. A virus specific association of PW and virions in mixed infections of wheat streak mosaic- and wheat spindle streak mosaic viruses [31] was suspected to have functional significance in respect of replicative processes. However, although monolayer aggregates of virions are often found at PW [22, 41, 49, 56, 57], they are also formed at various membranes of cell organelles like mitochondria, chloroplasts, peroxisomes and nuclei (Fig. 5A) or along tonoplast membranes [32]. In our studies we could not detect any association of specific NSP in close association to the PW or with such virion arrangements. NSP in dense, sometimes crystalline aggregates like PW are unlikely to be working as enzymes in replicative processes. The functional NSP should be expected non-aggregated in the cytoplasm of the infected cells in amounts of single or few molecules and most probably associated with membranes. Membrane-associated RNA replication complexes were theoretically expected [1, 51, 53, 60] and experimentally corroborated by replicative activity in crude membrane fractions [37].

The site of viral RNA replication could theoretically be indicated by simultaneous IGL detection of at least a helicase (CI) and a RNA-polymerase (NIb) at certain sites. With our methods both NSP were detected together only in the electron-dense nuclear inclusions of BtMV. A significance of the CI-NIb association in respect of replicative processes can in this case not be seen since membranes were not associated with BtMV-induced nuclear inclusions and because NSP associations like with BtMV were not detected by us with other potyviruses.

As likely candidates for membrane-associated replicative complexes on the ultrastructure level appear in our study the clusters of vesicles often found within complex accumulations of cellular organelles and NSP inclusions induced by potyviruses [32, 33]. Such membrane clusters might be homologous in function to those induced by comoviruses and picornaviruses [51]. For cowpea mosaic virus RNA replication has been demonstrated to proceed in cytopathic clusters of membraneous vesicles [21, 58].

Upon search for an experimental support for this hypothesis weak labelling by antisera to HC-Pro, NIa and NIb could be shown for the suspicious sites. But CI was not detected, which should additionally be expected because of its putative helicase function. However, low concentrated CI might have escaped our detection because the antiserum to CI had a relatively low reactivity. The antisera to purified PW of PVA and of PVY were available for us, but were known to have weak reactivities also to CP of the respective viruses and could, therefore, not be used for the detection of low amounts of CI protein.

For the interpretation of our results it should further be kept in mind that the potyvirus-infected tissues studied here were mainly from late stages of infection which presumably had already passed maximal viral replication activity [4]. Our studies of developing margins of local lesions indicated a very fast transition between early and late PPV infection stages on *Chenopodium quinoa* (results not shown here). Therefore, this study can report in detail on the composition of inclusions, but is of only limited value for evaluating the putative replication site. Preliminary results of in situ hybridization studies on ultrathin sections corroborated our interpretations since viral RNA in *sense* as well as in *antisense* orientation was detected in the vesicle-containing areas (to be published later on).

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