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Subcellular localization and immunodetection of movement proteins of olive latent virus 1

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Summary. Polyclonal sera raised to *Escherichia coli*-expressed movement proteins encoded by ORF 3 (p8K) and ORF 4 (p6K) of olive latent virus 1, were used for their immunodetection in infected *Nicotiana benthamiana* plants. In subfractionated locally infected tissues 4 days post inoculation (d.p.i.) that were analysed by Western blot, p8K was found in the fast-sedimenting fractions P1 and P30 containing membranous material and/or cell organelles and, likely, the fibrous structures mentioned below, but not in the soluble protein-containing supernatant. No p6K could be detected in these extracts. In locally inoculated leaves p8K began to accumulate from 2 d.p.i onwards reaching its peak at 4 d.p.i. Intracellular immunogold labelling of cells from locally and systemically infected tissues localized p8K primarily in fibrous inclusions made up of thin filaments with a helical structure present in the cytoplasm of locally and systemically infected cells. In systemic infections a light and scattered labelling was oberved in the cytoplasm and near the cell wall. The specific serum to p6K did not label the fibrous structures and failed to recognize its antigen in systemically and locally infected tissues except at 4 d.p.i., when scattered labelling was observed in the cytoplasm and near plasmodesmata.

Introduction

Olive latent virus 1 (OLV-1), is a definitive species within the genus *Necrovirus* (family *Tombusviridae*) reported from olive [10], citrus [23], and tulip [19]. The virus has isometric particles c. 30 nm in diameter, that assemble and accumulate, often in crystalline arrays, in the cytoplasm of infected cells [3]. The viral genome, a completely sequenced monopartite ssRNA [14], comprises five open reading

frames (ORFs) encoding polypeptides of 23, 82, 8, 6, and 30 kDa in the 5′–3′ direction. Site directed and deletion mutagenesis of infectious transcripts showed that the expression products of ORF1 (23 kDa protein) and ORF2 (82 kDa protein) are both required for virus replication, those of ORF3 (8 kDa) and ORF4 (6 kDa) are involved in cell-to-cell movement, whilst the 30 kDa coat protein (ORF5) assists in long distance translocation [27].

With this study, the subcellular localization of the 8 kDa (p8K) and 6 kDa (p6K) proteins was determined by cell fractionation, gold immunotagging, and by fusion with the green fluorescent protein (GFP), a marker widely used for identifying the cellular localization of viral proteins [5, 6, 12, 15, 17, 18, 29].

Materials and methods

Virus isolate, purification, and RNA extraction

The OLV-1 isolate, the same used in previous studies [3, 14, 27], was propagated in *Nicotiana benthamiana*, from which it was purified as previously described [14]. RNA was extracted from purified virus preparations by the SDS-phenol method [8], analysed by electrophoresis in 1.2% agarose, and stained with ethidium bromide.

Cloning in pGEX-6P vector, protein expression and purification

One μg of gel-purified viral RNA was used as template in a RT-PCR carried out as described [23]. Two primer pairs were designed for amplifying the coding region of the genes expressing the 8K (8Kfor and 8Krev1) and 6K (6Kfor and 6Krev1) proteins (Table 1). The expected DNA fragments, after digestion with *Bam*HI and *Eco*RI, were ligated into the pGEX-6P-1 plasmid

Table 1. Primer pairs used for PCR amplification of the coding sequences of OLV-1 movement proteins and the GFP5 gene

Name	Sequence	Position
8Kfor	5′-AAAGATCCATGGATTACCAAACTGAGGTT-3′*	nt 2215–2236
8Krev1	5′-AAAGAATTCCTAAAAGTTAAAGTTGTATGTCAC-3′	nt 2416–2440
6Kfor	5′-AAAGGATCCATGGCTGTGTGTCGCTGTTGT-3′	nt 2442–2463
6Krev1	5′-AAAGAATTCCTATTTTGCGATCGAAATGTGTTG-3′	nt 2589–2613
8Krev2	5′-AAACCGCGGAAAGTTAAAGTTGTATGTCAC-3′	nt 2416–2440
6Krev2	5′-AAACCGCGGTTTTGCGATCGAAATGTGTTG-3′	nt 2589–2613
GF1	5′-AAACCGCGGAGTAAAGGAGAAGAACTTTTCAC-3′	GFP specific
GF2	5′-TGTAGAGAGAGACTGGTGATTTC-3′	GFP specific
8KrevH	5′-ACCTCCACCTCCGGAAATTCCCGGCCCAAA GTTAAAGTTGTATGTCAC-3′	nt 2416–2440
6KrevH	5′-ACCTCCACCTCCGGAAATTCCCGGCCCTTTT 5GCGATCGAAATGTGTTG-3′	nt 2589–2613
GFHIN	5′-AATTTCCGGAGGTGGAGGTGGAATTCTAGATAG TAAAGGAGAAGAACTTTTCAC-3′	GFP specific

*Non viral sequences are underlined

(AP Biotech, USA) and cloned in *Escherichia coli* strain DH5α as previously described [13]. The recombinant clones, denoted pGEX-8K and pGEX-6K, were used to transform *E. coli* strain BL21. GST-8K and GST-6K fusion proteins were expressed and purified as described [16].

Antiserum production

Both fusion proteins were purified by affinity chromatography and concentrated by lyophilization. Approximately 300 μg of each protein were dissolved in 500 μl of phosphate-buffered saline (PBS), emulsified with an equal volume of incomplete Freund's adjuvant, and injected subcutaneously into rabbits. Four weekly booster injections of the same amount of fusion protein were given before bleeding. Crude sera did not react with healthy *N. benthamiana* extracts, and were routinely used at 1:1,000 dilution for all Western blot assays.

Preparation and analysis of plant protein extracts

Healthy and OLV-1-infected *N. benthamiana* leaves (400 mg) were homogenized in 1 ml of Laemmli's buffer [20] to obtain total protein extracts. Samples were boiled for 5 min and the insoluble material removed by centrifugation for 5 min at 15,000 g. For subcellular fractionation, tissue samples were collected from locally infected leaves 4 d.p.i., and processed as described [7]. The accumulation of p8K in the course of infection was investigated by fractionating extracts from tissues collected from locally infected leaves 12 and 18 h d.p.i., then daily up to the 6th d.p.i. Protein samples from total extracts and single fractions were resolved by electrophoresis on 12% polyacrylamide gels (PAGE). Western blots were done and visualized as described [15].

Electron microscopy

Electron microscope observations were carried out on inoculated leaves of *N. benthamiana* 2, 3, 4, 5, and 6 d.p.i. and on systemically infected leaves. Leaf tissue were cut to small fragments and processed according to standard procedure [22], i.e. fixation in 4% glutaraldehyde in 0.05 M phosphate buffer, post-fixation in 1% osmium tetroxide for 2 h at 4 °C and staining overnight in 2% aqueous uranyl acetate in the cold, dehydration in graded ethanol dilutions and embedding in Spurr' medium. Thin sections were stained with lead citrate before viewing with a Philips Morgagni 282D electron microscope. Controls consisted of healthy leaf tissue fragments processed as above. For immunogold labelling (IGL) fragments of *N. benthamiana* tissues were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, dehydrated in graded ethanol dilutions and embedded in London White resin. IGL was carried out essentially as described [4], using antisera to recombinant p8K and p6K, diluted 1:1,600 and 1:800, respectively, and a preparation of colloidal gold (15 nm in diameter) (Amersham, U.K.) conjugated with goat antirabbit antibodies. Controls consisted of sections of the same series as the labelled ones that were exposed to pre-immune sera diluted 1:400.

Construction and expression in protoplasts of OLV-1 MPs fusions with GFP5

Sequences coding for p8K and p6K were cloned in C-terminal fusion with the green fluorescent protein variant 5 gene (GFP5) [28]. Viral sequences were PCR-amplified using primer pairs 8Kfor/8Krev2 and 6Kfor/6Krev2, whereas primers GF1 and GF2 were used for amplifying the GFP5 gene (Table 1). The fragments corresponding to p8K and p6K were digested with *Sac*II, separately ligated with *Sac*II-digested GFP5 DNA and further digested with *Bam*HI and *Pst*I. After purification from agarose gel, the 8K::GFP5

and 6K::GFP5 fusion genes were cloned into the *Bam*HI and *Pst*I sites of pGY1 vector [26] downstream of the 35S promoter and the recombinant clones were denoted pS8KGFP and pS6KGFP.

To avoid a possible misfolding of OLV-1 MPs (8K, M_r 8,383 Da; 6K, M_r 6,264 Da) due to the contact with the much bigger GFP (M_r 26,844 Da), a sequence coding for 13 amino acids (GPGISGGGGGILD, referred to as H, personal communication by Dr. A. Giesel) was inserted in the joining point of the fusion protein (OLV1 MP C-terminus/GFP N-terminus) to work as an hinge. The stretch of five glycine residues, contained in the H sequence, should address the correct folding of the fusion proteins, by ensuring the rotation of the two portions of the chimeric product. The chimeric constructs were prepared by amplifying p8K and p6K coding sequences with 8Kfor/8KrevH and 6Kfor/6KrevH primer pairs, and the GFP5 gene using primers GFHIN and GF2 (Table 1). The 8KH::GFP5 and 6KH::GFP5 fusion genes were cloned into the *Bam*HI and *Pst*I sites of pGY1 vector and the obtained clones were denoted pS8KHGFP and pS6KHGFP.

N. benthamiana R1 protoplasts were isolated according to [24], cultured, rinsed, and transformed by PEG-mediated direct gene transfer as described [9, 25]. Ten micrograms of DNA were used for the transformation of *c.* 500,000 protoplasts. After 2 h, the protoplasts were rinsed, resuspended in 2 ml culture medium and incubated at 26 °C in the dark. Protoplasts were observed by fluorescence microscopy in their culture medium at 12, 18, and 24 h after transformation.

Confocal laser scanning microscopy

A Zeiss LSM5 Pascal confocal laser scanning microscope was used to obtain confocal images (objective 40.0/1.0 oil). Microscope filters for FITC and for Texas Red staining were used for detecting GFP fluorescence and chlorophyll red fluorescence, respectively. The images shown in Fig. 5. were produced using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Results

Presence and subcellular localization of OLV-1 MPs in N. benthamiana

Western blot analysis of total protein extracts from *N. benthamiana*-infected tissue failed to detect p8K and p6K (not shown), in agreement with what reported for *Turnip crinkle virus* [21], a member of the same family as OLV-1. When concentrated subcellular fractions [i.e., 1,000 g pellet (P1), 30,000 g pellet (P30), and 30,000 g supernatant (S30)] from healthy and locally infected leaves harvested 4 d.p.i. were exposed to the serum directed against GST-8K, which was able to detect p8K after protease digestion, p8K was found in the fast sedimenting fractions P1 and P30, but not in fraction S30 (supernatant containing soluble proteins) (Fig. 1A). By contrast, the specific GST-6K antiserum, which was able to detect the protease-digested GST-6K fusion protein, failed to detect a polypeptide of the expected size in any subcellular fraction from infected tissues (Fig. 1B). Thus, for time-course studies the accumulation of the sole p8K was investigated using the P30 fraction from locally-inoculated leaves. In these extracts, p8K became detectable from 2 d.p.i. till the end of the experiment (6 d.p.i.), reaching its maximum at 4 d.p.i. (Fig. 1C).

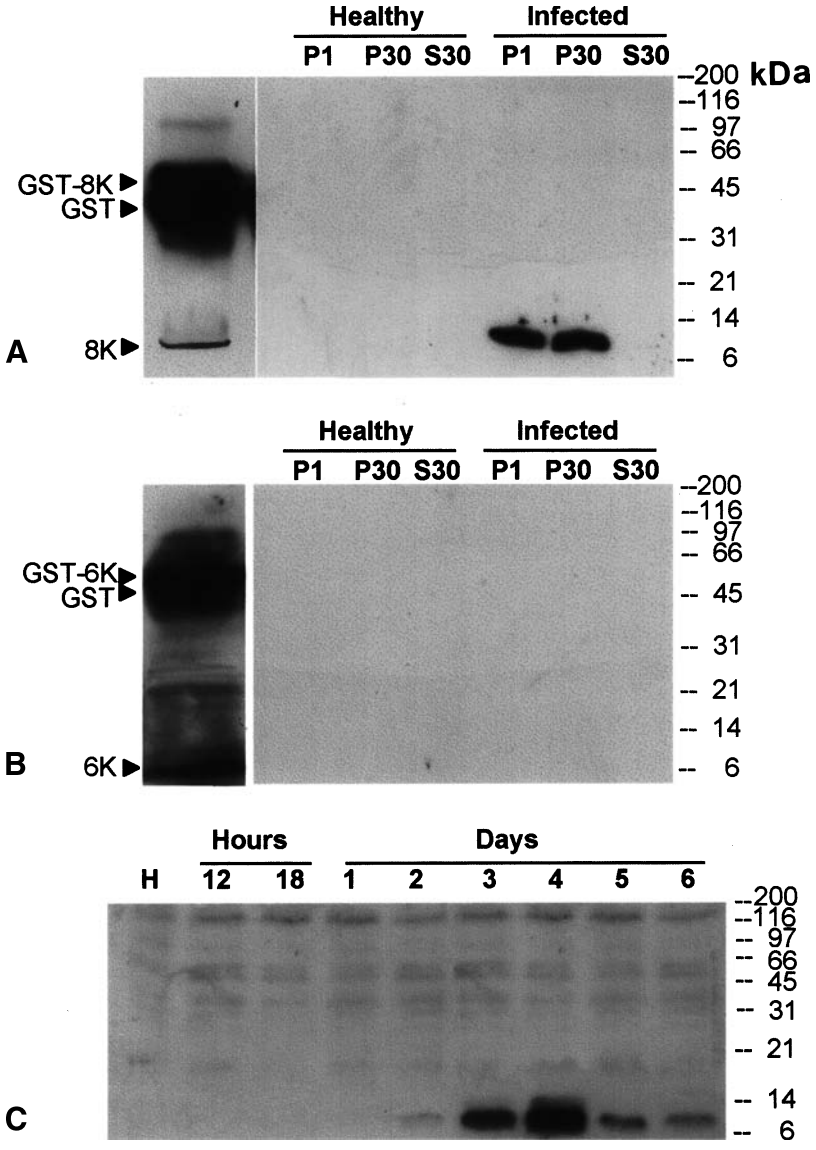

Fig. 1. **A**, **B**. Western blotting of subcellular fractions corresponding to 10 mg of healthy and locally inoculated *N. benthamiana* leaves. Whereas p8K (**A**) was absent or below detection level in the supernatant containing soluble proteins (fraction S30), it occurred in fraction P1 (1,000 g pellet containing primarily cell organelles) and P30 (30,000 g pellet containing primarily cytoplasmic membrane material). By contrast, p6K was not identified in any of the fractions (**B**) likely because of a concentration below the detection level of the system used. The prescission-protease-digested products of GST-8K and GST-6K expressed in *E. coli* are shown to the left of panel **A** and **B**, respectively, and protein position is indicated. **C** Proteins of fraction P30 corresponding to 10 mg of infected tissue were extracted at the time shown at the top of the figure. p8K was immunodetected with the specific serum from 2 d.p.i. onwards. *H*, healthy control. Molecular weight markers in kDa, are indicated on the right of the figures

Ultrastructure of locally and systemically infected N. benthamiana *cells*

Locally inoculated *N. benthamiana* leaves at 2 d.p.i. did not show apparent cytological alterations (not shown). These became visible at 3 d.p.i. in the form of small cytoplasmic aggregates of filamentous structures, membrane proliferation from the endoplasmic reticulum, and a few vesicular evaginations of the tonoplast containing fibrillar material. These alterations increased in severity as time passed (not shown).

Systemically infected tissues showed ultrastructural modifications conforming to those described in detail in [3], i.e. no visible damage to mitochondria and chloroplasts, extensive vesiculation of the nuclear envelope (Fig. 2A), abundant presence of virus particles in the ground cytoplasm either randomly scattered (Fig. 2A) or in small aggregates, and fibrous structures with a criss-cross pattern which, sometimes, had a tubular aspect (Fig. 2A, inset). As previously shown [3], these inclusions were in fact bundle-like aggregates of filaments with a helical structure, rather than tubules like those characterizing infections by nepo-, como-, faba-, and oleaviruses [15, 22]. Interestingly, comparable fibrous inclusions had been observed in cells infected by an isolate of *Tobacco necrosis virus* (TNV-W) [1], another member of the genus *Necrovirus.*

In situ localization of the 8K and 6K proteins

No labelling was observed in any of the locally infected control cells treated with pre-immune sera (e.g. Figs. 2D and 3C, inset). Likewise, there was no apparent tagging in locally infected cells exposed to p8K serum at 2 d.p.i. By contrast, at 3 d.p.i. a very light labelling was seen in the nuclei (Fig. 2B) and a much stronger labelling on the fibrous structures in the cytoplasm (Fig. 2C). Nuclei of cells at 4, 5, and 6 d.p.i. were no longer labelled, but tagging was abundant and largely restricted to the bundles of cytoplasmic filaments (Fig. 3A–C). The situation did not change in systemically infected tissues (Fig. 4B), except for the occasional labelling in proximity of the cell wall (Fig. 4B, inset). No labelling was seen in the inoculated locally infected leaves exposed to p6K antiserum, except for the very few and scattered gold particles detected in the cytoplasm or close to plasmodesmata of cells at 4 d.p.i. (Fig. 4A and inset). There was no tagging by the p6K serum of the cytoplasmic fibrous structures (Fig. 4A) in any of the cells observed, regardless of the age of the infection, thus confirming their p8K nature.

In vivo localization of the OLV-1 movement proteins fused with the GFP

Expression in *N. benthamiana* protoplasts of p8K-GFP 18 h after transformation was throughout the cytoplasm and the nucleus (Fig. 5A) like GFP alone (Fig. 5B). On the contrary, p6K-GFP appeared to be associated with the nuclear envelope and with structures next to the plasma membrane which may tentatively be identified

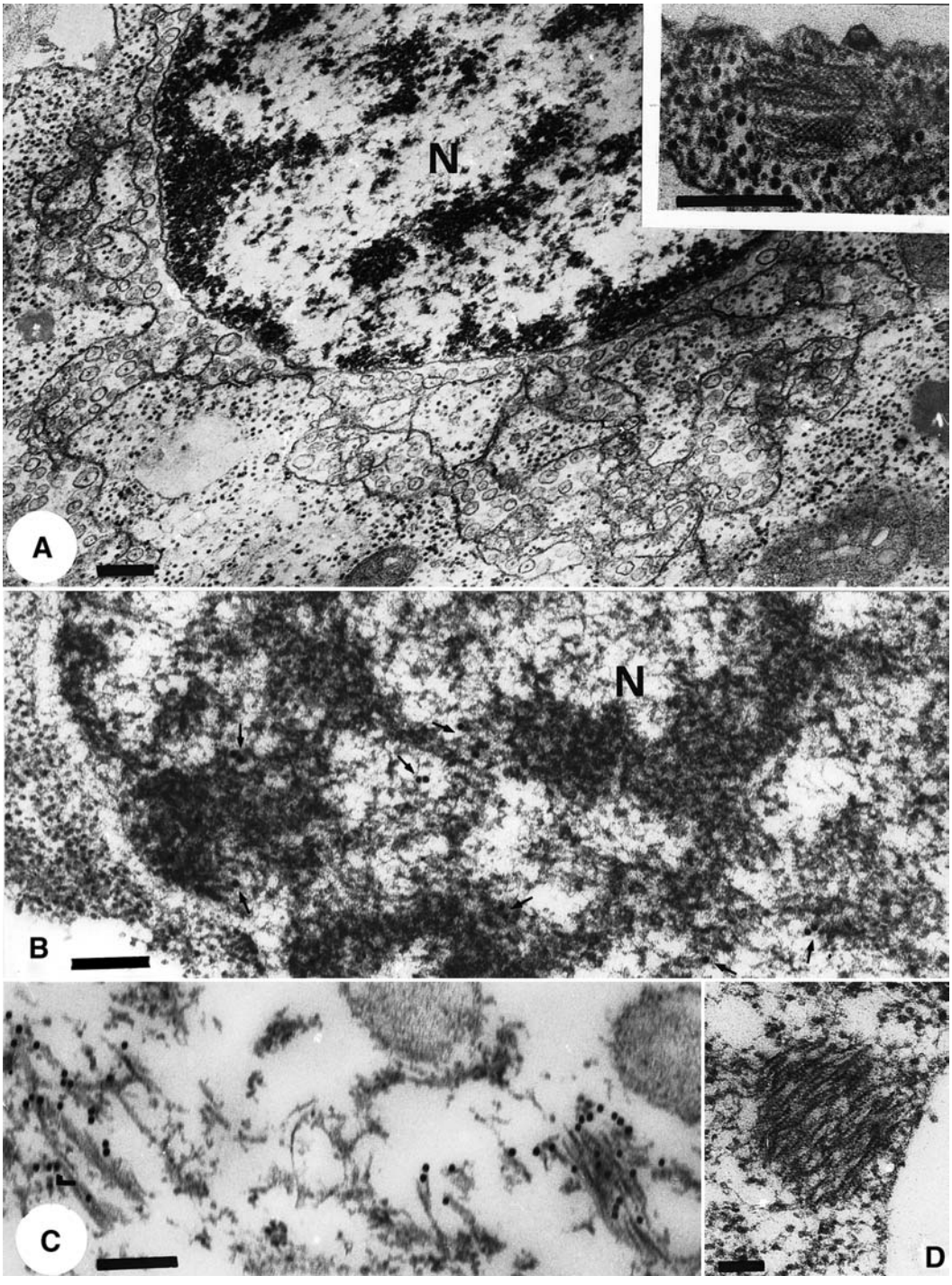

Fig. 2. A. Extensive vesiculation of the nucleus (*N*) of a *Nicotiana benthamiana* cell systemically infected by OLV-1, 12 days after inoculation. Inset shows a group of structures with a criss-cross pattern, somewaht resembling tubules. **B**. Extremely light and scattered labelling in a nucleus (*N*) of a locally infected *N. benthamiana* cell 3 d.p.i exposed to the serun to p8K. **C**. Cytoplasm of a locally infected cell 3 d.p.i. Labelling by the p8K serum is localized on bundles of filaments. **D**. shows filaments in an infected control cell treated with preimmune serum diluted 1:400 Bars = 200 nm

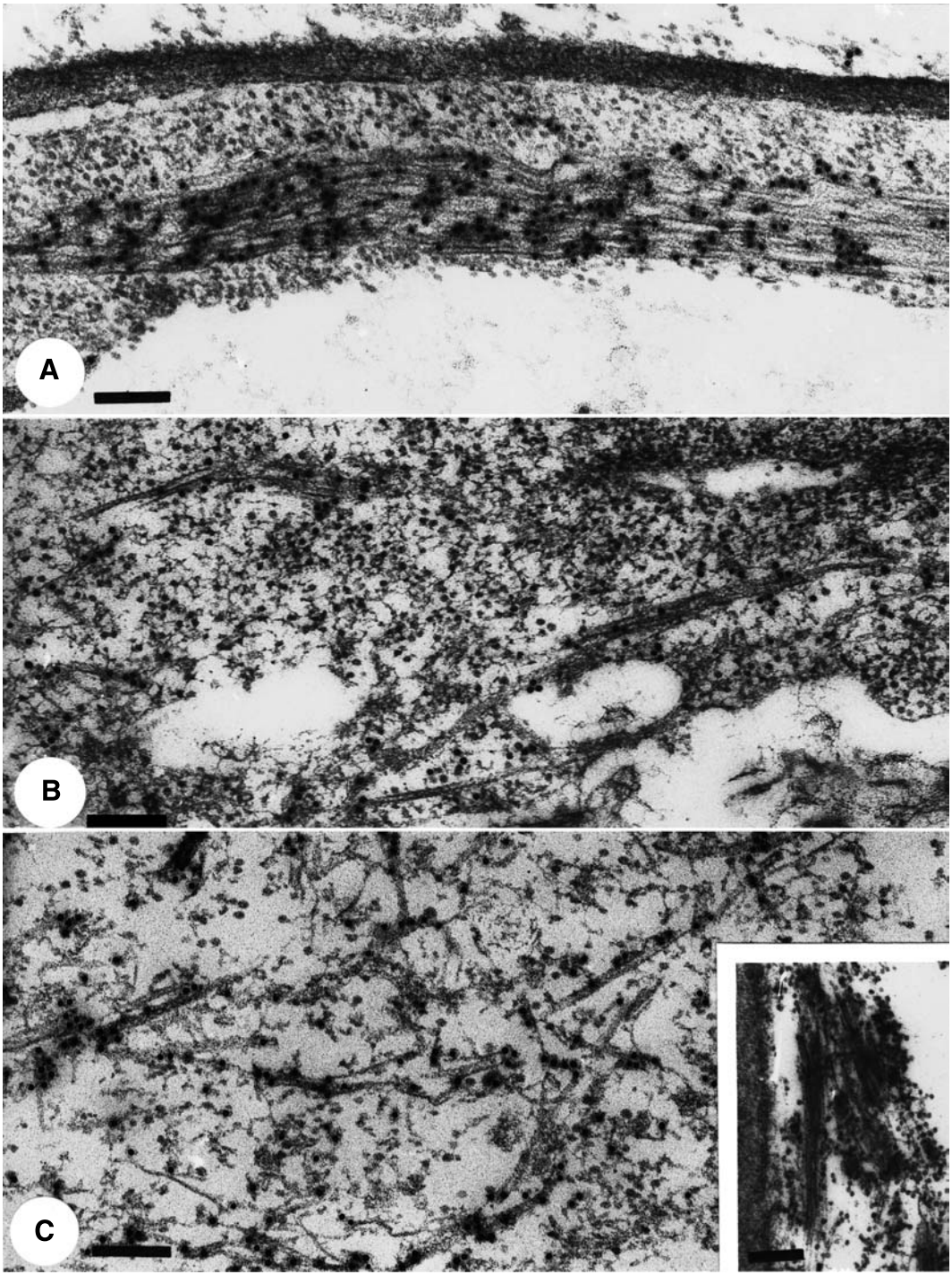

Fig. 3. Locally infected cells of *N. benthamiana* 4 (**A**), 5 (**B**), and 6 (**C**) d.p.i. In all cases, labelling by the p8K antiserum is largely restricted to the cytoplasmic fibrous structures. Inset in C shows filaments in a control cell. Bars = 200 nm

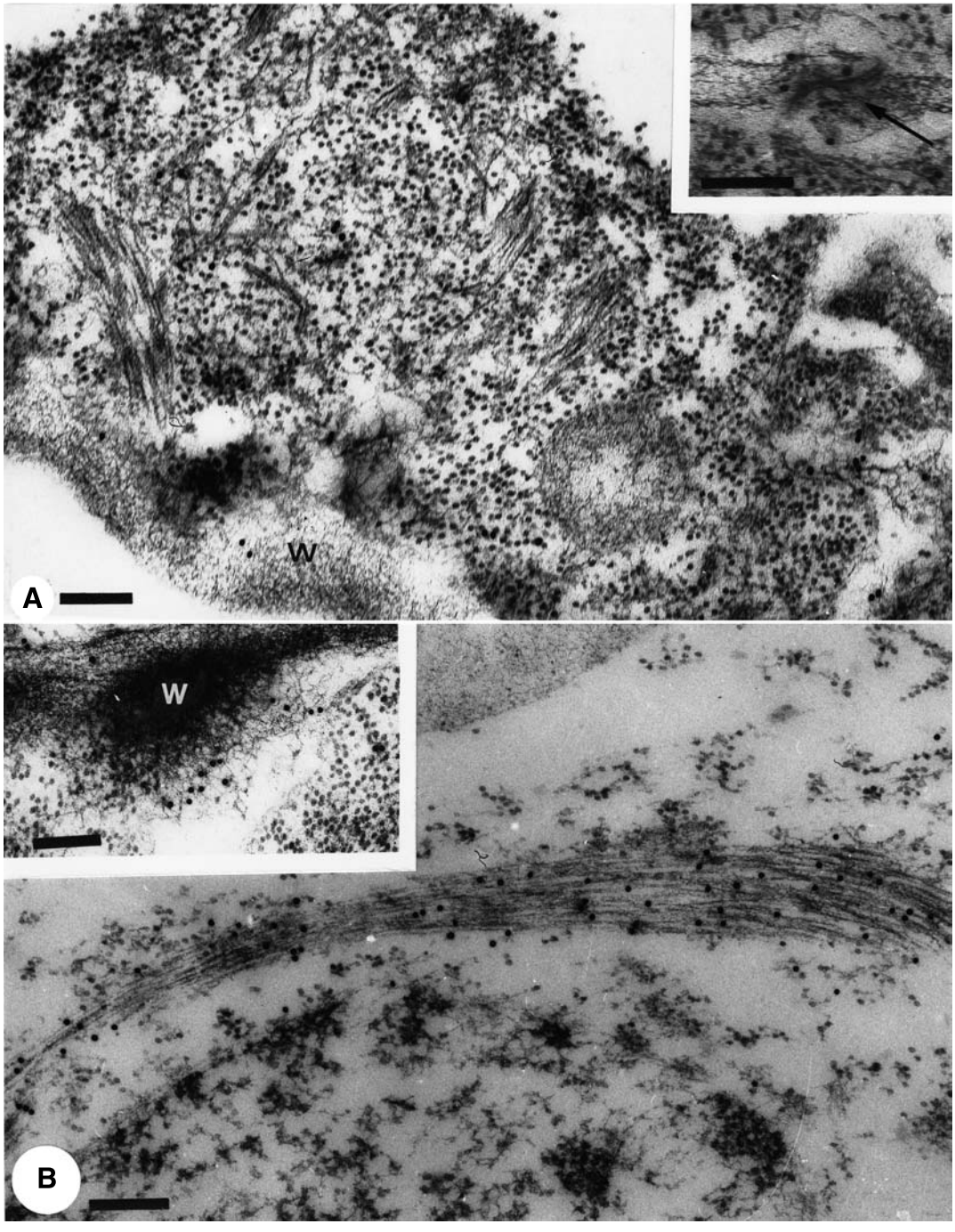

Fig. 4. A. Locally infected *N. benthamiana* cell 4 d.p.i. exposed to gold-labelled serum to p6K. Tagging is very light and scattered throughout the cytoplasm or close to plasmodesmata (arrow in inset) but in no case occurs on filamentous structures. **B**. Systemically infected *N. benthamiana* cell 12 days after inoculation exposed to gold-labelled serum to p8K showing tagging mostly concentrated on bundles of filaments and close to the cell wall (*W*). Bars = 200 nm

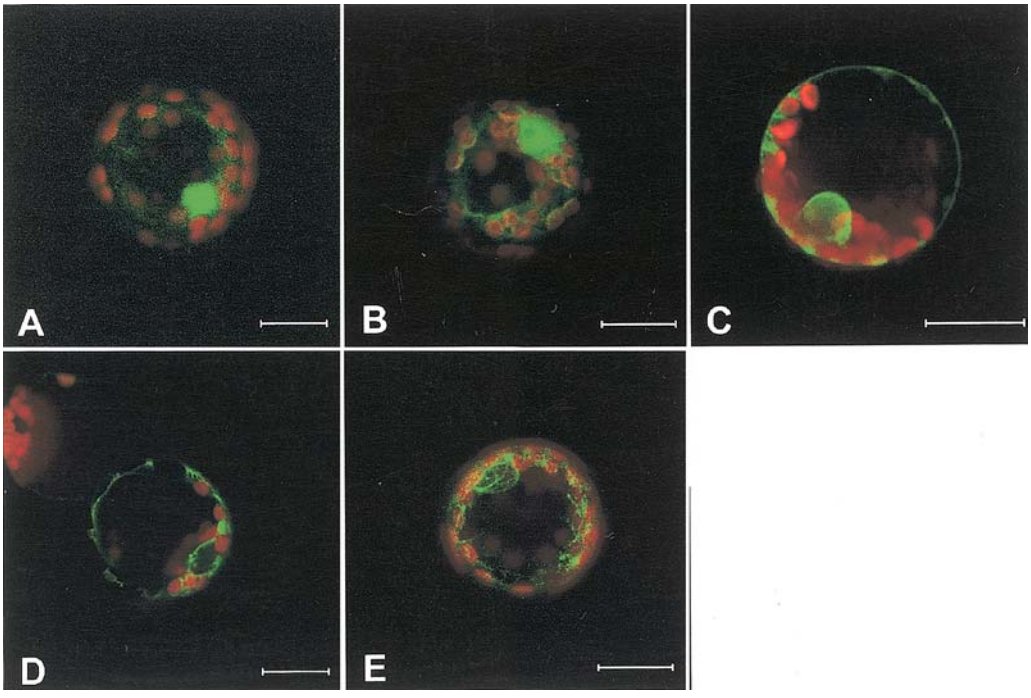

Fig. 5. Detection by CLSM of fluorescence of p8K-GFP and p6K-GFP transiently expressed in tobacco protoplasts and epidermal cells. Fluorescence distribution in protoplasts expressing p8K-GFP (**A**), GFP alone as control (**B**), p6K-GFP (**C**) and 6KH-GFP (**D**), 8KH-GFP (**E**). Chloroplasts fluoresce red. Bars = 20 μm

as endoplasmic reticulum (Fig. 5C). When the construct 6KH-GFP, that contained a sequence encoding 13 amino acids (GPGISGGGGGILD), inserted in the joining point of the fusion protein (OLV1 MP C-terminus/GFP N-terminus) to work as an hinge was expressed in *N. benthamiana* protoplasts, it showed the same localization as p6K-GFP (Fig. 5D), By contrast, 8KH-GFP fluorescence was no longer diffused as in Fig. 5A, but it was concentrated in punctuate structures, of variable size in the perinuclear region (Fig. 5E).

Discussion

As previously reported [27], OLV-1 p8K and p6K are involved in cell-to-cell virus movement, since their absence does not interfere with RNA replication, but prevents virus spreading from inoculated leaves. Both these proteins are translated from the 1518 bp bicistronic sgRNA, and may be expressed at different rates from one another, as reported for the movement proteins of TCV [21].

The present investigation has disclosed the localization of p8K *in vivo*, detecting it by Western blot analysis in the fast sedimenting cytoplasmic fractions P1 and P30. Besides membranes and organelles [7], these fractions are likely to contain

the cytoplasmic fibrous structures which, based on the results of immunogold labelling, appear to be aggregates of p8K. Since these aggregates apparently did not disassemble during subfractionation, for no visible trace of p8K was present in the supernatant liquid that contains soluble proteins, it is plausible to conclude that during extraction they mix with cell membranes and organelles, sedimenting with them. Thus, a great deal of p8K occurred in the cytosol in aggregated form. The similarity of OLV-1 fibrous structures with those observed in cells infected by TNV [1], suggests that also these latter inclusions may consist of aggregated p8K. If so, fibrous inclusions may represent a normal form of intracellular accumulation of necroviral p8K.

Although OLV-1 p8K accumulation levels decreased somewhat during late infection stages (6 d.p.i.) in locally infected leaves, its expression was basically non-transient, as reported for the comparable p7 protein of *Carnation mottle virus* (CarMV) [11], another member of the family *Tombusviridae*.

As to p6K, it could not be detected *in vivo,* except for the light labelling of locally infected cells at 4 d.p.i (Fig. 4A). This situation recalls that of p9 movement protein of TCV, which could not be detected *in vivo* because of the very low translation efficiency of its downstream ORF 3 [21].

The construct 8K-GFP did not localize specifically to any cellular compartment, similarly to GFP alone. However, the introduction of a sequence encoding 13 amino acids, which worked as an hinge in the joining point of the fusion protein, allowed the 8KH-GFP to localize in dispersed punctuate structures concentrated also in the perinuclear region. The OLV-1 p8K association with nuclei resembles that of the p8 movement protein of TCV, the difference being that contrary to the latter [6], the OLV-1 p8K presence in the nucleoplasm, based on the results of immunolabelling, seems to be transient.

Interestingly, the addition of the GFP at the C-terminus of the much smaller p6K did not apparently interfere with its subcellular localization, as shown by the similar fluorescence pattern obtained when 6K-GFP and 6KH-GFP were expressed in *N. benthamiana* protoplasts. The 6K-GFP appeared to be associated with the nuclear envelope and, possibly, the endoplasmic reticulum. If so, this is in line with observations on CarMV movement protein p9, which was shown to be an intrinsic membrane protein docking the endoplasmic reticulum without the intervention of other viral of host factors [30]. OLV-1 p6K retains only one predicted transmembrane motif (TM; AILILILAILVV), preceded by polar and proline residues, which may still be sufficient for a putative membrane-docking function, as suggested for *Galinsoga mosaic virus* and *Melon necrotic spot virus* [30], two additional members of the family *Tombusviridae*. Thus, the results of our study seem to be largely consistent with those reported for other members of the family *Tombusviridae* [2, 5, 6, 21, 30].

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