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Subcellular localization and *in vivo* identification of the putative movement protein of olive latent virus 2

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The gene encoding the 36·5 kDa ('36K') nonstructural protein located on RNA3 of olive latent virus 2 (OLV-2) was cloned, expressed with the *Escherichia coli* pGEX-2T system and the purified protein used to raise a polyclonal antiserum. Immunoblot analysis of OLV-2-infected *Nicotiana benthamiana* plants showed that the 36K protein accumulated in the early stages of infection and was associated with a subcellular fraction enriched in cytoplasmic membranes. In infected cells there were tubular structures, some containing virus-like particles, scattered in the cytoplasm or protruding from or penetrating the cell wall at the plasmodesmata. Immunogold labelling localized the 36K protein in the plasmodesmata of OLV-2-infected cells and showed it to be associated with virus-containing tubules. Leaf trichome cells of *N. tabacum* plants, transformed with a 36K–green fluorescent protein (GFP) fusion construct, revealed localized fluorescence in the cell walls, possibly due to association of the fusion protein with plasmodesmata. When the same 36K–GFP fusion protein was expressed in *N. tabacum* protoplasts, long tubular fluorescent structures protruded from the protoplast surface, suggesting that the 36K protein is responsible for tubule induction. The conclusion is drawn that this protein is likely to be the OLV-2 movement protein, mediating cell-to-cell virus movement, and that movement is by a tubule-guided mechanism.

Introduction

Olive latent virus 2 (OLV-2), the type species of the genus *Oleavirus* (family *Bromoviridae*), has quasi-spherical to bacilliform particles and a nonpolyadenylated, tripartite, positive-sense ssRNA genome. Virions encapsidate four major RNA species, which have been sequenced (Grieco *et al.*, 1995, 1996). RNA1 (3126 nt) and RNA2 (2734 nt) are both monocistronic molecules encoding replication-related proteins, with conserved helicase, methyltransferase (RNA1) and RNA polymerase (RNA2) motifs. RNA3 (2438 nt) encodes a 36·5 kDa protein, and the 20 kDa coat protein (CP). RNA4 (2078 nt), coterminal with RNA3, has no determined biological function. A subgenomic RNA (ca. 1042 nt) responsible for CP production is formed in infected plants but it may not be encapsidated (Martelli & Grieco, 1997).

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To move from one cell to another, plant viruses must traverse the cell wall through plasmodesmata (Maule, 1991; Deom *et al.*, 1992; Lucas & Gilbertson, 1994; Carrington *et al.*, 1996). Two mechanisms have been described for cell-to-cell movement of viruses, both associated with modifications of the plasmodesmal channel mediated by the virus-encoded movement protein (MP): (i) the tobacco mosaic virus (TMV)-like mechanism, where the virus moves in a nonvirion form through the plasmodesmata (Citovsky & Zambryski, 1991) and (ii) the cowpea mosaic virus (CPMV)-like mechanism, which involves formation of tubular structures transporting virus particles to the adjacent cell (van Lent *et al.*, 1990). The composition of the tubules and the pathway by which they are formed have not yet been clearly determined. However, MP was reported to be a structural element of the tubules for several viruses, such as CPMV (van Lent *et al.*, 1990), cauliflower mosaic (CaMV; Linstead *et al.*, 1988), red clover mottle (RCMV; Shanks *et al.*, 1989), tomato spotted wilt (TSWV; Storms *et al.*, 1995), grapevine fanleaf (GFLV;

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Ritzenthaler *et al*., 1995) and tomato ringspot (TRV; Wiecorek & Sanfaçon, 1993) viruses. Tubular projections were also observed on the external surface of infected protoplasts, i.e. structures that lack both cell wall and plasmodesmata (van Lent *et al*., 1990; Perbal *et al*., 1993; Storms *et al*., 1995; Ritzenthaler *et al*., 1995; Zheng *et al*., 1997). Notwithstanding the [2]complete molecular characterization of the OLV-2 genome, it is still not known how this virus moves from cell to cell. However, the 36·5 kDa protein was suggested to be the possible OLV-2 MP because of its similarity to the P3a movement protein of bromoviruses and cucumoviruses and the presence of the G/D motif typical of the 30K movement protein superfamily (Grieco *et al*., 1995). In this paper, the intracellular localization of the OLV-2 36·5 kDa protein (36K) and its ability to form tubular structures were determined, thus providing evidence that this protein is likely to be the OLV-2 MP.

Methods

■ **Virus isolate, purification and RNA extraction.** The OLV-2 isolate was the same as used in previous studies (Grieco *et al*., 1996). The virus was propagated in *Nicotiana benthamiana*, from which it was purified as previously described (Grieco *et al*., 1995). Nucleic acids were extracted from virus preparations fractionated on a sucrose gradient (Gonsalves & Shepherd, 1972), analysed by electrophoresis in 1·2% low-melting-point agarose and stained with ethidium bromide. The band corresponding to RNA3 was excised and the nucleic acid extracted (Sambrook *et al*., 1989).

■ **Cloning in pGEX-2T vector, protein expression and purification.** One µg of gel-purified OLV-2 RNA3 was used as template in an RT–PCR carried out as described by Martelli *et al*. (1996). The two deoxyprimers for PCR were designed to amplify the coding region of the 36K protein, and corresponded to nt 360–386 (3A2, 5′ AAAGGATCC ATGGCTGGTTTGTGGCGTTCTAACTCC 3′; forward) and nt 1352–1373 (3A1, 5′ AAAGGATCCTTATGTTTGACGCACCGGAGCG 3′; reverse) of the RNA3 OLV-2 sequence (nonviral sequences containing a *Bam*HI restriction site are underlined). The expected DNA fragment was digested with *Bam*HI, ligated into *Bam*HI-cut, dephosphorylated pGEX-2T plasmid (Pharmacia) and cloned in *Escherichia coli* strain DH5α as previously described (Grieco *et al*., 1992). The recombinant clone pGEX-3A, containing the insert in the correct orientation, was selected and used to transform *E. coli* strain BL21. Fusion protein was expressed and purified as described by Hay *et al*. (1994).

■ **Antiserum production.** GST–36K fusion protein was purified by affinity chromatography and concentrated by lyophilization. Approximately 300 µg of protein was dissolved in 500 µl of PBS and emulsified with an equal volume of complete Freund's adjuvant before subcutaneous injection into a rabbit. Four weekly booster injections of the same amount of fusion protein, emulsified with incomplete Freund's adjuvant, were given before bleeding. The crude antiserum was routinely used at 1 : 2000 for all Western blot assays. To adsorb antibodies reacting against healthy plant antigens, 5 µl of antiserum was added to 1 ml of healthy *N. benthamiana* extract (50 µg tissue/ml PBS). After shaking for 4 h at room temperature and overnight incubation at 4 °C, the preparation was centrifuged for 10 min at 15000 ***g*** and the supernatant used for Western analyses.

■ **Preparation and analysis of plant protein extracts.** Healthy or OLV-2-infected *N. benthamiana* leaves (400 mg) were homogenized in 1 ml of Laemmli's buffer (Laemmli, 1970) to obtain total protein extracts. The samples were boiled for 5 min and the insoluble material was removed by centrifugation for 5 min at 15000 ***g***. Subcellular fractionation of healthy and infected *N. benthamiana* tissues was carried out as described by Deom *et al*. (1990). Protein samples from total extracts and single fractions were resolved by electrophoresis on 12% polyacrylamide gels and Western blots were done as described by Grieco *et al*. (1995). Visualization of antigen–antibody complexes was by chemiluminescent assay (ECL, Amersham) done according to the manufacturer's instructions.

■ **Electron microscopy.** For ultrastructural studies tissue fragments were excised from young *N. benthamiana* leaves that were showing symptoms and processed according to standard procedures (Martelli & Russo, 1985). Embedding was in Spurr's resin after dehydration in graded ethanol dilutions. Thin sections were stained with lead citrate before viewing with a Philips 201C electron microscope. Controls consisted of healthy leaf tissue fragments processed as above. For immunogold labelling (IGL), fragments from symptomatic leaf tissue of *N. benthamiana* were fixed in 2% glutaraldehyde in 50 mM phosphate buffer, thoroughly rinsed in the same buffer, dehydrated in graded ethanol dilutions and embedded in London White resin. IGL was carried out essentially as described by Faoro *et al*. (1991). The antiserum to OLV-2 36K protein, preadsorbed as above and diluted 1 : 1000, and a preparation of colloidal gold (15 nm diameter) conjugated with anti-rabbit antibodies (Amersham) were used. Thin sections were viewed with a Philips 201C electron microscope.

■ **Construction of pSGFP5–3A and transfection of protoplasts.** To clone the 36K protein gene in C-terminal fusion with the green fluorescent protein variant 5 gene (GFP5; Siemering *et al*., 1996) the two genes were PCR-amplified using the 36K protein gene oligoprimers 3A2 (forward) and 3A3 (5′ AAACCGCGGTGTTT-GACGCACCGGAGCG 3′; reverse) and the GFP5 gene oligonucleotides GF1 (5′ AAACCGCGGAGTAAAGGAGAAGAACTTTTCAC 3′; forward) and GF2 (5′ TGTAGAGAGAGACTGGTGATTTC 3′; reverse) (nonviral sequences containing a *Sac*II restriction site are underlined). The resulting fragments were digested with *Sac*II, ligated with T4 DNA ligase and further digested with *Bam*HI and *Pst*I. After purification from agarose gel, the 36K–GFP5 fusion gene was cloned into the *Bam*HI and *Pst*I sites of pGY1 vector (Neuhaus *et al*., 1991) downstream of the 35S promoter; the recombinant clone was designated pS3AGFP. *N. tabacum* R1 protoplasts were isolated following the protocol of Nagy & Maliga (1976), cultured and rinsed using the indicated media and transformed by PEG-mediated direct gene transfer as described (Freydl *et al*., 1995; Negrutiu *et al*., 1987). Ten µg of pS3AGFP was used for the transformation of ca. 500000 protoplasts. After 2 h, the protoplasts were rinsed, resuspended in 2 ml of culture medium and incubated at 26 °C in the dark. Protoplasts were observed by fluorescence microscopy in their culture medium at different times after transformation.

■ ***N. tabacum* transformation with pBIN-3agfp5.** The fused 36K–GFP gene was excised from pSGFP-3A by cutting with *Bam*HI and *Sac*I and the agarose gel-purified DNA fragment was cloned into the analogous restriction sites of the pBINm-gfp5er vector (Haseloff *et al*., 1997). The recombinant clone was denoted pBIN-3agfp5. *Agrobacterium tumefaciens* GV3101 was transformed by triparental mating using *E. coli* HB101(pRK2013) helper strain. Tobacco seedlings were grown for 4–6 weeks on MS classic medium (Calbiochem) at 25 °C under continuous light and cut stems were transferred to fresh MS boxes. Leaf pieces, 3–6 cm in length, were inoculated with transformed *A. tumefaciens* GV3101 in liquid MS, placed onto MSS (MS, 3% sucrose, 1 mg/l 6-

benzylaminopurine, pH 5·8) plates and kept at 25 °C for 4 days, before transfer to MSS plates containing antibiotics (100 μg/l kanamycin, 400 μg/l cefotaxime) which were incubated at 25 °C for 4–6 weeks. Shoots ca. 3 cm long were first transferred to MS medium with 50 mg/l kanamycin, then to soil.

■ **Confocal laser scanning microscopy.** A Leica DMR confocal laser scanning microscope and the LeicaTCS 4D operating system were used to obtain confocal images (objective 40·0/1·0 oil). Microscope filters for FITC and for Texas Red staining were employed for detecting GFP fluorescence and chlorophyll red fluorescence, respectively. The images shown in Fig. 3 were produced using Adobe Photoshop on a Power Macintosh 6500/275.

Results

Presence and subcellular localization of the OLV-2 36K protein in *N. benthamiana*

In extracts from *N. benthamiana* infected tissue a protein with an apparent molecular mass of 41 kDa was immunodetected; this protein was not present in healthy plant controls (Fig. 1A, arrowhead). This protein first became detectable 18 h post-inoculation (p.i.), reaching a maximum 3 days p.i (Fig. 1A). No protein bands were detected when the blots were probed with the preimmune antiserum (not shown).

Western immunoblots of different subcellular fractions (P1, P30 and S30), extracted from healthy and locally infected leaves (harvested 5 days p.i.), were analysed for the presence of the 36K protein. A protein of apparent molecular mass 41 kDa was found in the P30 fraction from infected but not healthy plants (Fig. 1B). This fraction primarily contained cytoplasmic membrane material (Deom *et al*., 1990).

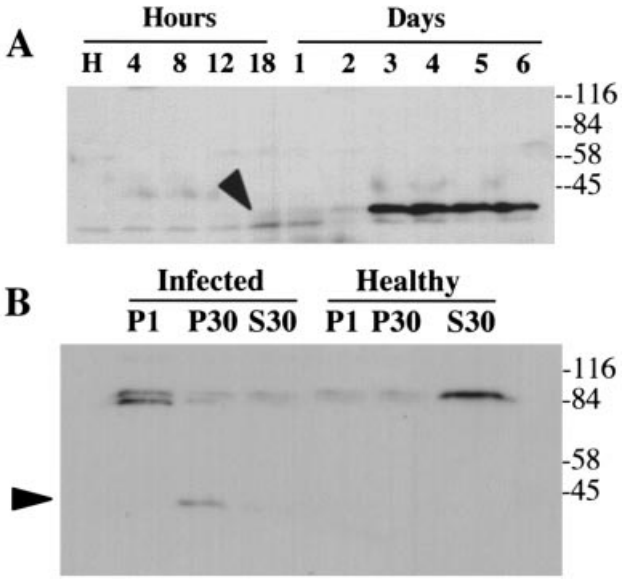

Fig. 1. (A) Kinetics of accumulation of the OLV-2 36K protein in inoculated leaves of *N. benthamiana*. Total proteins, corresponding to 10 mg of tissue from inoculated leaves, were extracted at the time indicated at the top of the figure and the 36K protein was immunodetected with specific antiserum. H, healthy control. (B) Immunodetection of 36K protein in subcellular fractions corresponding to 10 mg of healthy and OLV-2-inoculated *N. benthamiana* leaves. P1, 1000 ***g*** pellet; P30, 30000 ***g*** pellet; S30, 30000 ***g*** supernatant. Molecular mass markers are on the right of the panels. Arrowheads indicate the position of the 36K protein.

Ultrastructure of infected *N. benthamiana* cells

Infected *N. benthamiana* cells, but not healthy control cells, showed ultrastructural modifications conforming to those described in detail by Castellano *et al*. (1987). Cell walls showed localized thickenings, protrusions centred on plasmodesmata, and frequent paramural bodies. Tubular structures with variable length and a diameter of ca. 40 nm were present in the cytoplasm of many cells, either scattered or connected with plasmodesmata. Some of these structures contained rows of electron-dense round-to-ovoid bodies, interpreted as profiles of virus particles (Fig. 2A) (see also Castellano *et al*., 1987).

In situ localization of the 36K protein

Immunogold labelling was seen in infected but not in healthy *N. benthamiana* cells. Major organelles (i.e. nuclei, chloroplasts and mitochondria) were not labelled. In contrast, strong labelling was present in the cell walls near plasmodesmata (Fig. 2B), some of which showed the branched architecture typical of secondary plasmodesmata (Ding *et al*., 1992), and in clumps of densely staining material, occasionally seen in the cytoplasm (Fig. 2C). Many of the virus-containing tubules present in infected cells were also clearly labelled, some along the entire length (Fig. 2F) or, more commonly, at one or both extremities (Fig. 2D, E). No signal was detected when preimmune serum was used (not shown).

When viewed with a confocal microscope, leaf trichomes of *N. tabacum* SR1 transformed with a 36K–GFP fusion construct showed a specific fluorescence on the cell wall separating two adjacent cells (Fig. 3A, B).

The OLV-2 36K protein induces tubules in *N. tabacum* protoplasts

Protoplasts transformed with the OLV-2 36K–GFP fusion gene were observed under the confocal laser scanning microscope every 2 h post-transfection (p.t.). Fluorescence appeared 6 h p.t. within the interior of the cell. The intensity of fluorescence increased with time, so that 8–12 h p.t. the plasma membrane became fluorescent and, shortly afterwards (12–14 h p.t.), fluorescent tubular structures appeared at the protoplast surface (Fig. 3D). These structures grew to reach an average length of 20 μm, with a maximum length of 80 μm at 16 h p.t. (Fig. 3C, D). However, the tubules were apparently fragile, breaking off readily so that they no longer persisted at the protoplast surface 18 h p.t., although many were seen in the culture medium. No variations in fluorescence pattern were

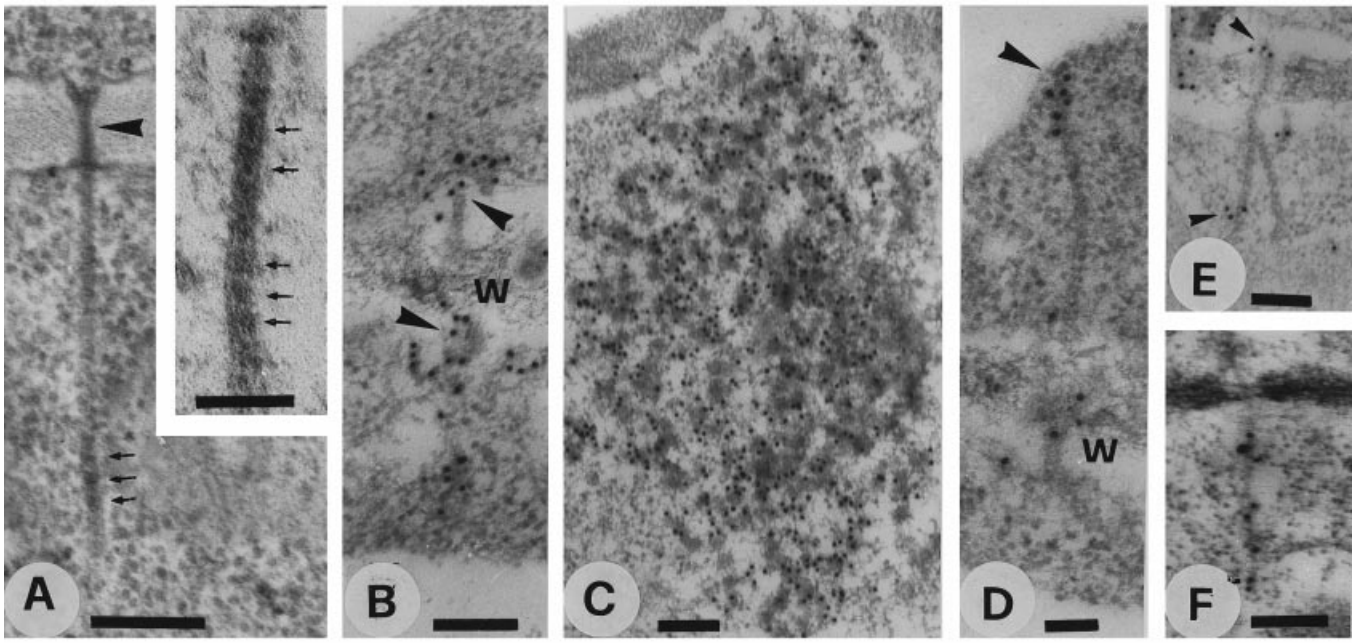

Fig. 2. Electron microscopy of infected *N. benthamiana*. (A) A virus-containing tubule (arrows) associated with a plasmodesma (arrowhead). Bar, 200 nm. Inset, part of a tubule at higher magnification showing profiles of virus-like particles (arrows). Bar, 100 nm. (B) Immunogold label at the level of plasmodesmata (arrowheads) in a systemically infected leaf. W, cell wall. Bar, 200 nm. (C) Heavily labelled clumps of electron-opaque material interpreted as possible accumulations of excess 36K protein in the cytoplasm of a systemically infected cell. Bar, 200 nm. (D) Tubular structure possibly associated with a plasmodesma showing gold labelling at one extremity (arrowhead). W, cell wall. Bar, 100 nm. (E) Tubular structure with both ends labelled (arrowhead) traversing a plasmodesma. Bar, 200 nm. (F) Tubular structure with labelling along the whole length. Bar, 200 nm.

observed in the protoplasts in the following 48 h. Interestingly, more than 90% of the tubules fluoresced more strongly at their extremities, thus showing a distribution of the 36K–GFP protein comparable to that observed by gold immunolabelling in the intracellular virus-containing tubules.

In protoplasts transfected with the vector expressing GFP alone, no tubules were formed and the fluorescence was more or less equally distributed in the cell rather than localized as in the protoplasts containing the 36K–GFP construct. This is taken as evidence that the 36K protein is responsible for both membrane targeting and induction of tubular structures.

Discussion

The results of this study, taken together with other observations, all support the idea that the 36K product of the gene located upstream of the CP cistron in OLV-2 RNA3 is an MP. Among the lines of evidence supporting this conclusion are the following. (i) The 36K protein has the conserved motifs of the '30K superfamily' of MPs (Grieco *et al.*, 1995). (ii) The 36K protein is an early product of translation, like the MPs of many plant viruses, including members of the family *Bromoviridae* to which OLV-2 belongs, e.g. alfalfa mosaic virus (AMV; Berna *et al.*, 1986) and cucumber mosaic virus (Vaquero *et al.*, 1996). (iii) The 36K protein has a subcellular localization consistent with that of MPs of a number of plant viruses (McLean *et al.*, 1993). In fact, the 36K protein was found in a cytoplasmic membrane-enriched fraction, and was detected by immunogold labelling in close proximity to or within plasmodesmata that lacked desmotubules, as recently reported also for AMV (van der Wel *et al.*, 1998). The plasmodesmal localization of the 36K protein was confirmed by fluorescent confocal microscopy of leaf trichomes of tobacco plants transformed with a 36K–GFP fusion construct, in line with the notion that viral MPs localize in the plasmodesmata of MP-transgenic plants (Ding *et al.*, 1992; Moore *et al.*, 1992; Vaquero *et al.*, 1996). (iv) The 36K protein is transiently expressed in transfected tobacco protoplasts. In these it was first observed in cell membranes, including the nuclear envelope, as observed for the MP of CPMV (van Lent *et al.*, 1991), and then in tubular structures protruding from the protoplast surface (van Lent *et al.*, 1991; Perbal *et al.*, 1993; Storms *et al.*, 1995; Ritzenthaler *et al.*, 1995), the formation of which it apparently elicits, as with TSWV (Storms *et al.*, 1995), CaMV and CPMV (Kasteel *et al.*, 1996). (v) It is associated with virus-containing tubules in infected plant cells.

Interestingly, immunoblot analysis showed that the apparent molecular mass of OLV-2 MP was ca. 41 kDa, i.e. significantly larger than the value (36 kDa) predicted from the amino acid sequence of the polypeptide (Grieco *et al.*, 1996). A

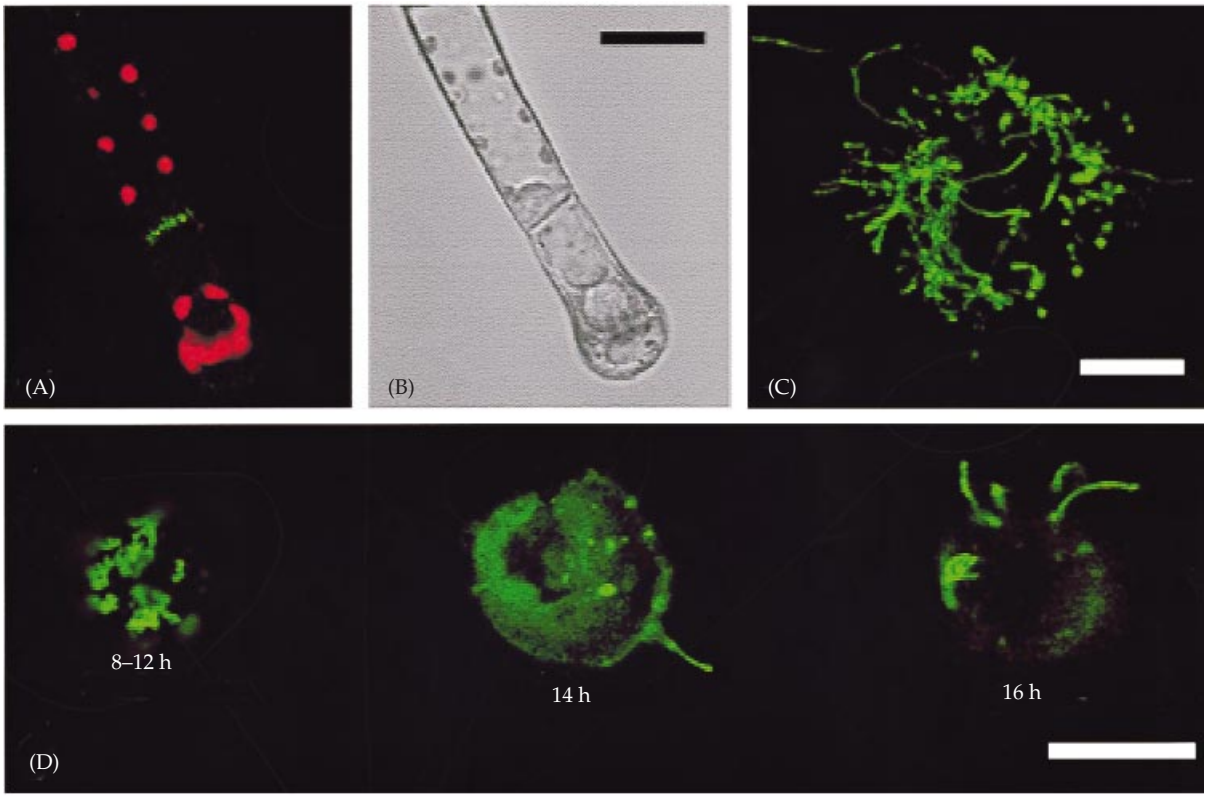

Fig. 3. (A) Confocal microscopy of leaf trichome of *N. tabacum* transformed with the 36K–GFP fusion gene, showing extensive green fluorescent structures in the cell wall between adjacent cells. Chloroplasts fluoresce red. (B) Bright field image of the trichome shown in (A). Bar, 20 μm. (C) An *N. tabacum* protoplast expressing the OLV-2 36K–GFP fusion gene 16 h after transfection. Note the abundance of fluorescent tubules protruding from the protoplast surface. Bar, 20 μm. (D) Confocal microscopy at different times after transfection of *N. tabacum* protoplasts expressing the OLV-2 36K–GFP fusion gene. Bar, 20 μm.

similar discrepancy, which may be explained as being caused by post-translational modifications, was also observed with MPs of CPMV (Wellink *et al.*, 1986) and RCMV (Shanks *et al.*, 1989).

OLV-2 is not the only member of the family *Bromoviridae* to induce the formation of virus-containing tubules. Comparable structures were previously observed in cells infected with tobacco streak (Martelli & Russo, 1985) and tomato aspermy (Francki *et al.*, 1985) viruses. Such tubules may constitute a common but transient feature (which is thus difficult to perceive) of *Bromoviridae* infections. Their detection in protoplasts (Zheng *et al.*, 1997) but not in tissue (Martelli & Russo, 1985) infected by AMV supports this notion. In the case of OLV-2, the tubules are abundant and readily seen. Immunogold labelling and fluorescent confocal microscopy have established that the tubules contain OLV-2 MP, but whether they are composed of this protein only remains to be ascertained.

In most cases, gold labelling was distinctively present at one or both extremities of the tubules, as with squash leaf curl virus (Ward *et al.*, 1997), and fluorescence was most intense at their base and tip. However, diffuse fluorescence occurred along the whole length of the tubules and, occasionally, a comparable pattern of gold labelling was observed. Such a distribution of the markers (GFP and colloidal gold) would be compatible with a possible localization of the 36K protein inside the tubular structure. This would make the MP accessible to gold-labelled antibodies when the tubules are parallel to the plane of sectioning, and their lumen is exposed. The accumulation of the MP at the tubule's extremities may confer a polarity to these structures, which could be important for the role they are thought to play in virus transport. Finally, although the intracellular distribution of OLV-2 MP was mostly linked with structures involved in virus transport (i.e. plasmodesmata and tubules), discrete clumps of gold-labelled material, possibly representing accumulations of excess 36K protein, were occasionally seen in the cytoplasm of infected cells.

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