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A protoplast system for studying tomato spotted wilt virus infection

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A plant protoplast system for studying tomato spotted wilt tospovirus (TSWV) infection was established and tested. Using polyethylene glycol-mediated inoculation with highly infectious TSWV particles, generally 50% or more of *Nicotiana rustica* protoplasts were infected. In these cells viral RNA and viral protein synthesis became detectable at 16 h post-inoculation (p.i.) and continued at least until 90 h p.i. Both the structural viral proteins [nucleoprotein (N) and the envelope glycoproteins G1 and G2] and the nonstructural viral proteins NSs and NSm accumulated to amounts sufficient for detection and immunocytological analysis. Local lesion tests on petunia leaves and electron microscopical analysis confirmed the production of mature, infectious virus particles, underlining the conclusion that a full infection cycle was completed in this system. Upon inoculation of *Vigna unguiculata* (cowpea) protoplasts with TSWV particles, comparable proportions of infected cells and amounts of NSs, NSm and N protein were obtained, but much lower amounts of viral glycoproteins were detected than in *N. rustica* protoplasts, and progeny virus particles were less abundant. With the *N. rustica*-based protoplast system, a powerful synchronized single-cell infection system has now become available for more precise *in vivo* studies of the processes occurring during tospovirus infection.

Introduction

Over the past few years a considerable amount of data on the molecular biology of tospoviruses, a genus of plant-infecting bunyaviruses, has been published (reviewed in: German *et al*., 1992; Goldbach *et al*., 1992; van Poelwijk *et al*., 1996; Mumford *et al*., 1996; Goldbach & Peters, 1996). These studies, which mainly focused on tomato spotted wilt virus (TSWV), the type species of the genus, revealed that tospoviruses have a tripartite RNA genome typical of all *Bunyaviridae*. The genomic RNAs are tightly associated with nucleoprotein (N) and enclosed by a lipid envelope from which the viral glycoproteins (G1 and G2) protrude, thus forming virus particles 80–110 nm in diameter. Sequence and translational analyses have shown the large (L) genomic RNA of TSWV to be negative-stranded (de Haan *et al*., 1991) and the other two (S and M) RNAs to be ambisense [de Haan *et al*. (1990) and Kormelink *et al*. (1992*a*), respectively]. In total six viral proteins appear to be specified by the genome of TSWV. The single translation product of L RNA (referred to as L protein) represents the putative viral polymerase (de Haan *et al*., 1991; van Poelwijk *et al*., 1993). The M RNA encodes, in its viral (v) strand, a common precursor to both envelope glycoproteins and, in its viral complementary (vc) strand, a nonstructural protein, NSm, implicated in cell-to-cell movement of the virus (Kormelink *et al*., 1992*a*, 1994; Storms *et al*., 1995). The (smallest) S RNA encodes the N protein in the vc strand and an additional nonstructural protein, NSs, whose function remains unknown, in the v strand (de Haan *et al*., 1990; Kormelink *et al*., 1991).

The TSWV infection cycle has so far only been studied in whole plants. An alternative would be to develop a protoplast infection system. Due to better synchronization of infection and, in general, high proportions of infection, single-cell suspensions have been of great value in the study of a considerable number of plant viruses. No such system has been described for tospoviruses, but high proportions of infection

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for *Vigna unguiculata*, *Nicotiana edwardsonii* and *Nicotiana benthamiana* protoplasts inoculated with Sonchus yellow net rhabdovirus (SYNV) have been reported (van Beek *et al*., 1985; Jones & Jackson, 1990), showing that such an approach is feasible for enveloped plant viruses.

This report describes the development of a protoplast system for TSWV, based on *Nicotiana rustica* and *V. unguiculata*, involving polyethylene glycol (PEG)-mediated inoculation with purified virus particles.

Methods

Virus and plants. A Brazilian isolate of TSWV, BR01 (de Ávila *et al*., 1993), was used, and maintained in *N. rustica* plants by mechanical inoculation and transmission by thrips.

Polyclonal antisera. Polyclonal antibodies were raised, as described previously, against the NSs (Kormelink *et al*., 1991), NSm (Kormelink *et al*., 1994) and N (de Ávila *et al*., 1993) proteins. Antibodies against the viral glycoproteins were raised by immunization of rabbits with purified fragments of G1 and G2, expressed in *E. coli* using the pET11t system (Novagen).

Isolation and storage of TSWV particles. Complete virus particles were isolated at 4 °C from systemically infected *N. rustica* leaves, essentially as described by Gonsalves & Trujillo (1986), with some modifications. Harvested leaves were ground in 3 ml of extraction buffer (0·01 M sodium sulphite; 0·1 M sodium phosphate pH 7·0) per g leaf material in a Philips blender by giving five to ten short pulses at medium speed. The homogenate was filtered through cheesecloth and the extract was centrifuged at 10000 ***g*** for 15 min. The pellet obtained was gently homogenized for 30 min in 1 ml 0·01 M sodium sulphite per g initial leaf material using a small pestle and a magnetic stirrer. The suspension was clarified by centrifugation at 8000 ***g*** for 15 min and the supernatant was collected and centrifuged at 100000 ***g*** for 30 min. The resulting pellet was homogenized in 5 ml 0·01 M sodium sulphite per 100 g of initial leaf material and 2·5 ml was layered per 10–40% sucrose gradient. After centrifugation for 45 min at 70000 ***g*** in a Beckman SW28 rotor, the opalescent zone containing virus particles was collected, diluted 1:1 with 0·01 M sodium sulphite and concentrated by centrifuging for 1 h at 100000 ***g***. The pellet, containing virus particles, was resuspended in sterile double-distilled water at a concentration of approximately 1 mg/ml. In some experiments the sucrose gradient at the end of the procedure was omitted, and the preparation was homogenized in sterile double-distilled water. This also yielded infectious virus suitable for inoculation of protoplasts. The amount of viral protein from each isolation was estimated using a Bio-Rad protein assay kit according to the manufacturer's procedure. Virus (= protein) yield was usually between 0·3 and 1 mg per 100 g of leaves. The preparations were kept on ice and used immediately, or promptly frozen in liquid nitrogen and stored at −80 °C. Before use, samples were then thawed slowly on ice.

Preparation of protoplasts. Protocols for isolation of protoplasts were based on those described by Hibi *et al*. (1975) and van Beek *et al*. (1985), and modified to some extent for *N. rustica*. Protoplasts of *N. rustica* were prepared from both greenhouse- and *in vitro*-grown plants, although the latter usually gave better results. *In vitro*-grown *N. rustica* plants were initiated by sterilization of seed, using bleach and SDS, and germination on sterile solidified Murashige and Skoog medium including vitamins (Murashige & Skoog, 1962), antibiotics and a fungicide (cefotaxime, 50 mg/l; vancomycin, 50 mg/l; nystatin, 25 mg/l respectively, all from Duchefa biochemicals, the Netherlands). Seedlings were first transferred to growth tubes (Sigma), or immediately to 'Vitro Vent' growth boxes (Duchefa biochemicals) with solidified medium and grown for 2·5 to 6 weeks. After removal of the midrib, leaves were transversally incised to generate a fine 'comb', and floated, lower epidermis down, on an enzyme solution containing 0·6 M mannitol, 10 mM $CaCl_2$ (pH 5·6), and 1% cellulase (Onozuka R-10) and 0·05% Macerozyme (both from Yakult Honsha Co., Japan). After incubation for about 3·5 h at 30 °C in the dark, with gentle shaking every hour, protoplasts were harvested by gentle shaking, collected, and put through a 64-mesh sieve. They were washed with 0·6 M mannitol, 10 mM $CaCl_2$ (pH 5·6) by centrifuging at 50 ***g*** for 3 min and subsequent resuspension; this was repeated three times. Preparations with less than 70% of living cells, as judged by fluorescein diacetate (FDA) fluorescence, were discarded.

V. unguiculata (cowpea) protoplasts were isolated from primary leaves of plants grown on liquid Hoagland medium (van Beek *et al*., 1985). The lower epidermis was stripped and the leaves were incubated with the stripped side down. The rest of the procedure was as described for *N. rustica*.

Inoculation of protoplasts with purified TSWV. The inoculation was based on protocols described by van Beek *et al*. (1985) and Eggen *et al* (1989). Glass tubes containing 10^6 living protoplasts (pelleted) were put on ice. Approximately 10 μg of ice-cold virus was added and the mixture was gently shaken. Subsequently, 0·5 ml cold 40% PEG, 10 mM $CaCl_2$ was added and the tube was shaken more vigorously for 15 s to mix well. To this, 4·5 ml 0·6 M mannitol, 10 mM $CaCl_2$ (pH 5·6) (room temperature) was then added and the tube inverted three times. The protoplasts were incubated for 15–20 min at room temperature after which they were washed two or three times with 0·6 M mannitol, 10 mM $CaCl_2$ (pH 5·6). After washing, the cells were resuspended in 2 ml of nutrition medium [0·6 M mannitol (pH 5·6), 2·5 mM MES (pH 5·6), 0·2 mM KH_2PO_4, 1·0 mM KNO_3, 1·0 mM $MgSO_4$, 10 mM $CaCl_2$, 1 mM KI, 0·01 mM $CuSO_4$, 25 mg/l nystatin, 50 mg/l vancomycin and 50 mg/l cefotaxime], and transferred to small polystyrene Petri dishes (3·5 cm diameter, Nunc) in a climate chamber at constant temperature (25 °C), constant light (about 5000 lx) and constant high humidity (> 70%). As a negative control aliquots of 10^6 protoplasts were inoculated with virus that was inactivated with 213 nm UV light for 10 min (4·8 J/cm^2) using a UV tray commonly used for analysing ethidium bromide stained gels.

Western immunoblot analysis. Protoplasts were pelleted by centrifugation at 50 ***g*** for 3 min, the supernatant was removed and 1 vol. of homogenization buffer (50 mM Tris–acetic acid pH 8·2, 10 mM potassium acetate, 1 mM EDTA, 5 mM DTT and 1 mM PMSF) was added. Subsequently, 1 vol. of 4× Laemmli buffer (Laemmli, 1970) was added and prior to electrophoresis the samples were boiled for 3 min. Five μl of the samples was loaded on a 10% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred onto PVDF membrane (Millipore) using the Bio-Rad Trans-Blot semi-dry blotting procedure. Filters were blocked overnight in 3% Elk skim instant milk in PBS containing 0·1% NP40. After washing with 0·3% instant milk, 0·1% NP40 in PBS, filters were incubated with 0·5 μg/ml polyclonal antisera against N, NSs or glycoproteins respectively for 1 h at room temperature. After washing, antigen–antibody complexes were detected using 1 μg/ml alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Tago Inc.) and a mixture of nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) as a substrate.

RNA isolation and Northern blot analyses. Aliquots of 10^6 protoplasts were pelleted, the supernatant removed, and the pellet frozen

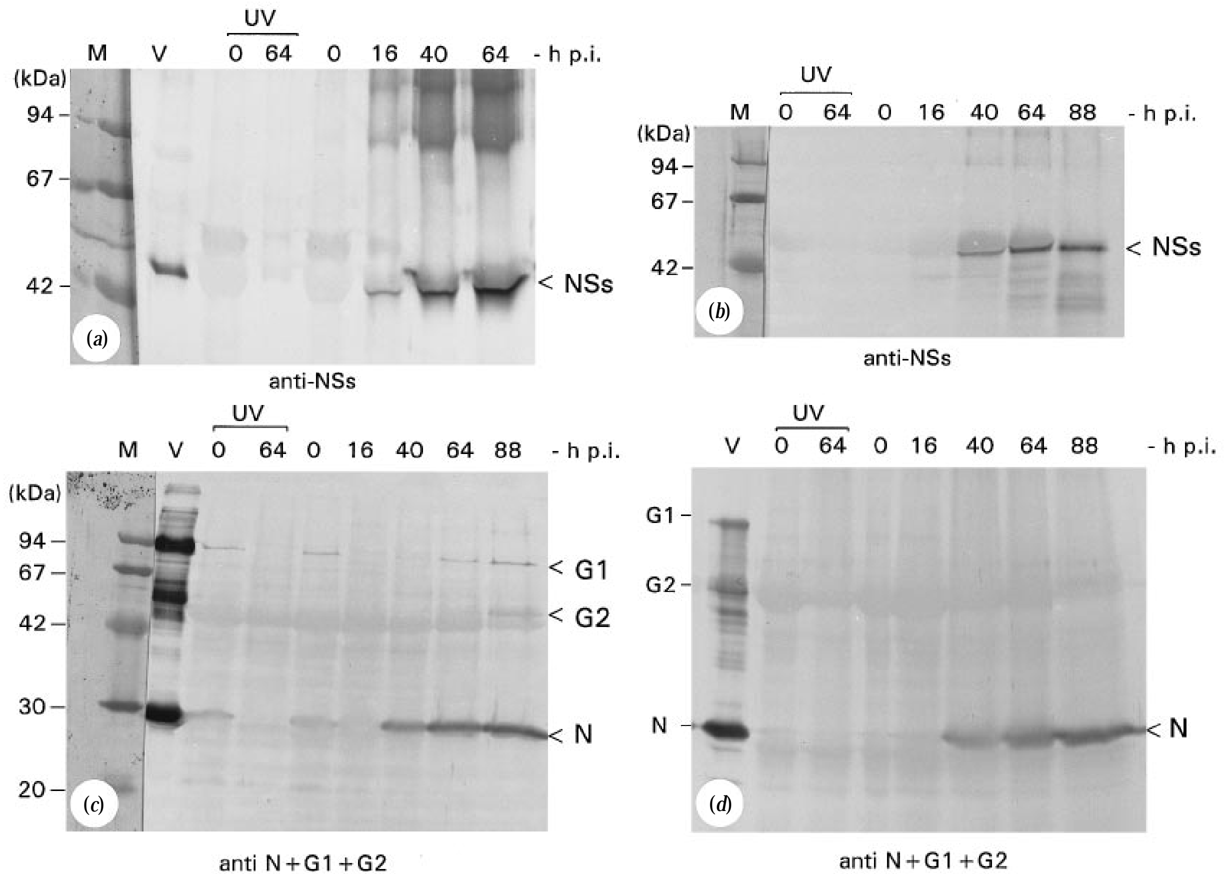

Fig. 1. Western blot analysis (using anti-NSs serum) of TSWV-inoculated *V. unguiculata* and *N. rustica* protoplasts at different times (h p.i.) showing production of NSs protein in *N. rustica* protoplasts (*a*) and *V. unguiculata* protoplasts (*b*); Western blot analysis (using a mixture of antisera against N, G1 and G2) showing production of N, G1 and G2 protein in *N. rustica* protoplasts (*c*) and production of N protein, but not G1 and G2, in *V. unguiculata* protoplasts (*d*). M, molecular mass markers; V, purified TSWV. Protoplast samples in (*a*) and (*c*), lanes 3 and 4, and in (*b*) and (*d*) lanes 2 and 3 (marked 'UV'), were inoculated with UV-inactivated virus.

in liquid nitrogen and stored at −80 °C. Total RNA was isolated using the GIBCO BRL TRIzol method. In brief, pellets were thawed and resuspended in 200 µl TRIzol reagent. After 5 min, 40 µl chloroform was added, the suspension mixed well and centrifuged for 15 min at 21 000 ***g*** in an Eppendorf centrifuge. The aqueous phase was transferred to a fresh tube containing 100 µl isopropanol. The RNA was pelleted, washed, dried and resuspended in 35 µl double-distilled water. Five µl samples were resolved in a 1% agarose gel after treatment with methylmercuric hydroxide (Bailey & Davidson, 1976). The RNA was blotted onto a Genescreen filter (New England Nuclear), and hybridized to ^{32}P-labelled riboprobes of TSWV-specific sequences (Kormelink *et al.*, 1992*b*). As a control, total RNA of TSWV-infected *N. rustica* plants was included.

■ **Immunofluorescence of inoculated protoplasts.** After incubation, protoplasts were spotted onto poly-L-lysine (0·05%)-treated glass plates, blocked with 5% BSA in PBS pH 7·2 and subsequently incubated with 1–5 µg/ml of antiserum against NSs, NSm, N or the viral glycoproteins in 1% BSA in PBS for up to 1 h at room temperature. Antigen–antibody complexes were detected using fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit serum (0·02 mg/ml, 45 min), and examined with a fluorescence microscope. At least 200 cells were judged for specific fluorescence to calculate infection percentages, using an antiserum against NSs.

■ **Local lesion tests on petunia leaves.** The infectivity of newly synthesized viral products was analysed on leaves of petunia (cv. Polo Blauw), a local lesion host of TSWV. Petunia plants were put in the dark 2 days before being used, to increase their susceptibility to infection. Leaves were cut from the plant and subsequently cut through the midvein. After dusting with carborundum 500 powder, one half of each leaf was inoculated with purified virus as an internal control, and the other half with an amount of a pelleted and disrupted (vigorously shaken) protoplast suspension equivalent to 200 000–500 000 living cells. Lesions were counted 4 or 5 days later.

■ **Electron microscopy.** Protoplasts were pre-fixed in 0·75% (w/v) glutaraldehyde (GA) in nutrition medium for 30 min at room temperature, and then fixed with 3% GA, 2% paraformaldehyde (PA) in phosphate–citrate (PC) buffer (0·1 M Na_2HPO_4 and 9·7 mM citric acid pH 7·2) for at least 1 h. The fixed cells were washed several times with PC buffer, resuspended in 200 µl PC buffer, and layered onto 500 µl 5% (w/v) liquid gelatin in PC buffer. Tubes were centrifuged at 50 ***g*** and excess gelatin was removed. After solidification pellets were cut in smaller pieces and kept in fixative (3% GA/2% PA) at 4 °C. Samples were dehydrated with ethanol and embedded in LR Gold without additional fixation to produce specimens for immunocytological analysis. General ultrastructural analysis was done on samples fixed additionally in 1% (w/v) osmium tetroxide

in PC buffer, stained with 1% (w/v) uranyl acetate, and dehydrated and embedded in LR White.

Results

TSWV inoculation of *N. rustica* and *V. unguiculata* protoplasts

To test their suitability for a single-cell infection system, freshly prepared *N. rustica* and *V. unguiculata* (cowpea) protoplasts were inoculated with purified TSWV using PEG. The percentage of protoplasts infected was estimated by immunofluorescence, using an antiserum against NSs, a nonstructural protein only produced after replication of the genome (de Haan *et al.*, 1990), and a reliable indicator of infection. The proportion of infected protoplasts was calculated as numbers of NSs-positive cells relative to total numbers of living cells. Survival of TSWV-inoculated cells was similar for *N. rustica* and *V. unguiculata* protoplasts, and decreased from at least 75% at 0 h post-inoculation (p.i.) to a minimum of 50% at 90 h p.i. In repeated experiments, generally 50% or more of both *N. rustica* and *V. unguiculata* protoplasts were infected, while synthesis of NSs became detectable as early as 16 h p.i. (see also Western blot results below). Highly infective inoculum, needed to reach such values, was best maintained by using virus preparations immediately after isolation. Freezing of purified virus in liquid nitrogen and slow thawing on ice still gave 80% of the original infectivity, but storage of virus at −80 °C for more than a week led to a significant drop of infectivity, making results of protoplast inoculations less reproducible.

Viral protein and RNA synthesis

Using polyclonal antibodies, the synthesis of viral structural and nonstructural proteins was followed in *N. rustica* and *V. unguiculata* protoplasts. The results showed that from about 16 h p.i. onward, NSs was clearly detected in both systems (Fig. 1*a*, *b*). At about the same time, production of the nucleoprotein (N) took place in both species, after an apparent partial degradation of the proteins of the inoculum during a latency period (Fig. 1*c*, *d*). Viral glycoproteins (G1 and G2) were detected in *N. rustica* protoplasts (Fig. 1*c*), but surprisingly, in *V. unguiculata* protoplasts, these proteins did not accumulate appreciably (Fig. 1*d*).

Production of viral RNAs after inoculation of *N. rustica* protoplasts is shown in Fig. 2. After a latency period of about 16 h, increasing amounts of full-length v and vc RNA were produced, as well as subgenomic-sized RNA species that, according to their size, most probably represent viral mRNAs. The results are in accordance with the production of viral proteins as described above. When probed for L-RNA, specific smaller products were observed (Fig. 2*c*), indicating the presence of defective interfering (DI) RNAs (Resende *et al.*, 1991). Protoplasts inoculated with UV-inactivated virus did not show any increase in the amount of viral protein (Fig. 1*a*, *c*,

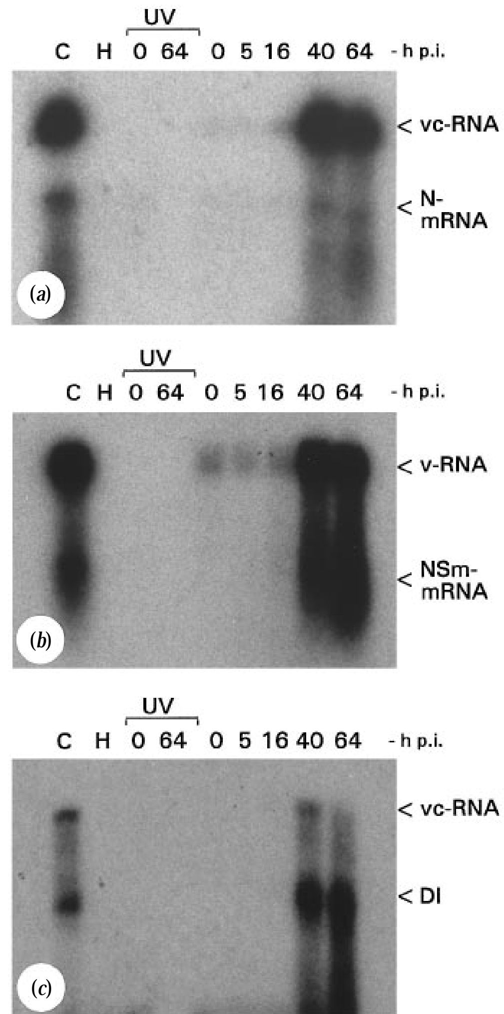

Fig. 2. Production of viral RNA species in *N. rustica* protoplasts at different times after TSWV inoculation (h p.i.), shown on Northern blots using riboprobes as described by Kormelink *et al.* (1992*b*), and specific for (*a*) full-length S-RNA (vc-sense; 2·9 kb) and N mRNA (0·9 kb), (*b*) full-length M-RNA (v-sense; 5·0 kb) and NSm mRNA (1·0 kb) and (*c*) full-length L-RNA (vc-sense; 8·9 kb). C, control (i.e. total RNA from infected *N. rustica* plants); H, total RNA from healthy *N. rustica* protoplasts. As a negative control, protoplast samples were inoculated with UV-inactivated virus (lanes marked 'UV'). DI, defective interfering RNA.

lanes 3 and 4; Fig. 1*b*, *d*, lanes 2 and 3) or viral RNA (Fig. 2*a*, *b*, *c*, lanes 3 and 4).

Immunofluorescence studies of inoculated *N. rustica* protoplasts

Using immunofluorescence microscopy, the intracellular location of viral proteins produced during protoplast infection was analysed. The results for *V. unguiculata* protoplasts were very similar to those for *N. rustica*, except for the lack of viral glycoprotein production; this paragraph therefore focuses on the results with *N. rustica*. Using antisera against N, G1 or G2, the inoculum virus was shown to adhere to the outside of the protoplasts at 0 h p.i. (Fig. 3*a*, *c*), whereas at times later than

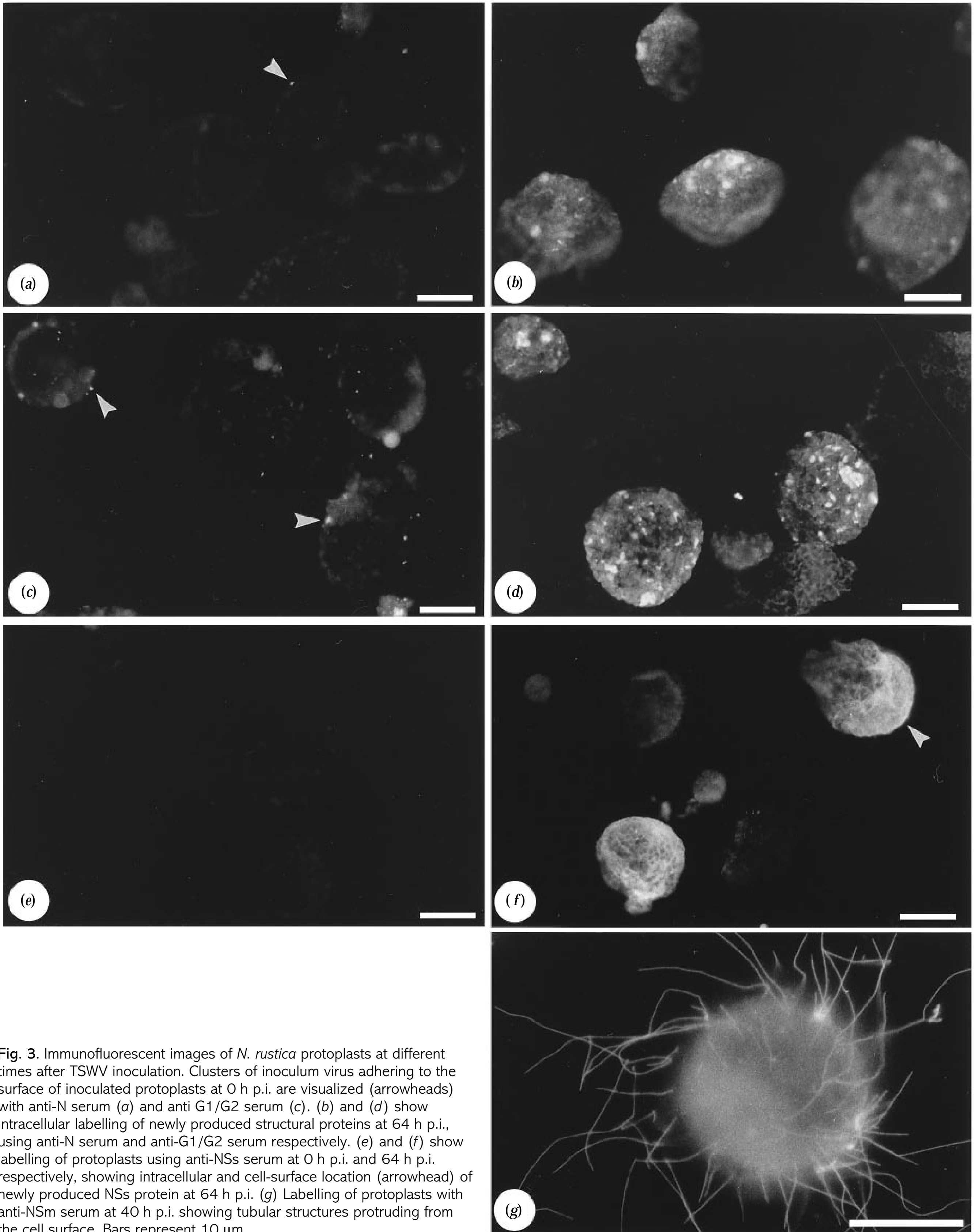

Fig. 3. Immunofluorescent images of *N. rustica* protoplasts at different times after TSWV inoculation. Clusters of inoculum virus adhering to the surface of inoculated protoplasts at 0 h p.i. are visualized (arrowheads) with anti-N serum (*a*) and anti G1/G2 serum (*c*). (*b*) and (*d*) show intracellular labelling of newly produced structural proteins at 64 h p.i., using anti-N serum and anti-G1/G2 serum respectively. (*e*) and (*f*) show labelling of protoplasts using anti-NSs serum at 0 h p.i. and 64 h p.i. respectively, showing intracellular and cell-surface location (arrowhead) of newly produced NSs protein at 64 h p.i. (*g*) Labelling of protoplasts with anti-NSm serum at 40 h p.i. showing tubular structures protruding from the cell surface. Bars represent 10 µm.

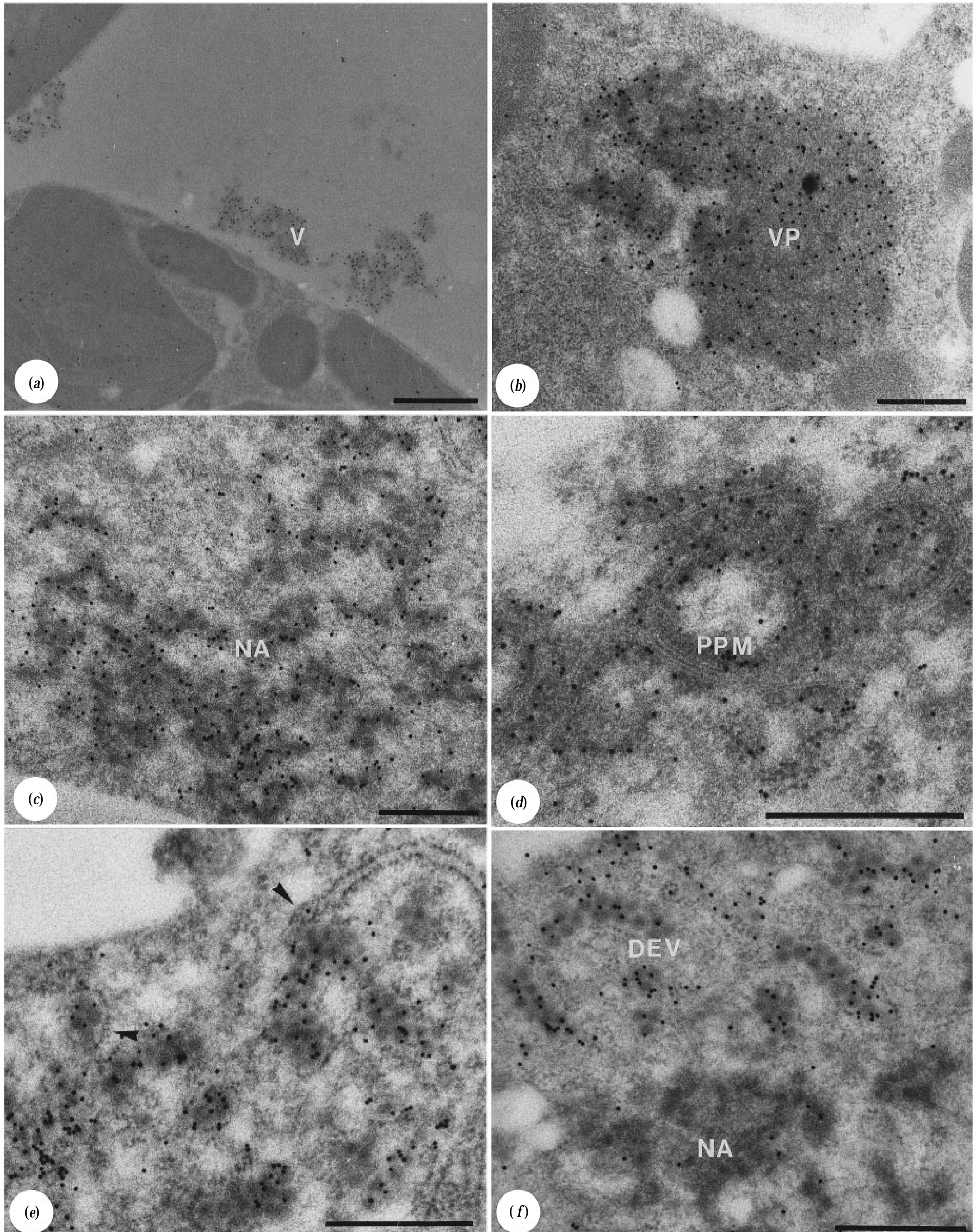

Fig. 4. For legend see facing page.

16 h p.i. internal production of the structural viral proteins became evident (Fig. 3*b*, *d*). The structural proteins appeared to be located in patches (Fig. 3*b*, *d*) whereas NSs (Fig. 3*f*) was more dispersed throughout the cytoplasm. *De novo* synthesized N, G1 and G2 were never seen at the cell surface, whereas NSs did appear at the cell surface later in infection (Fig. 3*f*). The disperse occurrence of NSs in the cytoplasm matches results of previous studies (Kitajima *et al*., 1992) which, for isolate BR-01, showed that NSs does not form fibrous aggregates, as it does in some other TSWV isolates.

The second nonstructural protein of TSWV, NSm, could also be monitored in infected *N. rustica* protoplasts. Kormelink *et al*. (1994) and Storms *et al*. (1995) have shown that this protein is involved in cell-to-cell translocation of the virus. In infected tissue NSm is targeted to plasmodesmata and subsequently aggregated into (plasmodesmata-penetrating) tubules (Storms *et al*., 1995). Although protoplasts do not, of course, have plasmodesmata, NSm appears to be correctly addressed in these cells. Immunofluorescent analysis of *N. rustica* protoplasts 40 h p.i. revealed the presence of long, NSm-containing tubules which extend from the surface of the cell (Fig. 3*g*).

Electron microscopy

Immediately after inoculation, only a few clusters of inoculum virus were recognized at the periphery of *N. rustica* (see Fig. 3*a*, *c*) and *V. unguiculata* protoplasts (Fig. 4*a*). Later, viroplasm and nucleocapsid aggregates that could be labelled with antibodies against N were observed in the cytoplasm of inoculated *V. unguiculata* protoplasts (Fig. 4*b*, *c*), but very few enveloped particles were found (data not shown). This low incidence of enveloped particles in *V. unguiculata* protoplasts fits the observation that in this cell system viral glycoprotein synthesis is hampered (Fig. 1*d*). In *N. rustica* protoplasts, however, enveloped progeny virus was frequently found in the cytoplasm, often associated with endoplasmic reticulum membranes, starting from 16 h p.i. and increasing with time (Fig. 4*e*, *f*). This confirms our earlier conclusion that in this system the TSWV infection cycle is efficiently completed. In addition, other cytopathological structures characteristic of TSWV infection (Kitajima, 1965; Francki & Grivell, 1970; Milne, 1970; Ie, 1971; Kitajima *et al*., 1992; Lawson *et al*., 1996) were also encountered in inoculated *N. rustica* protoplasts: e.g. viroplasm (exemplified for *V. unguiculata* in Fig. 4*b*), nucleocapsid aggregates (Fig. 4*f*) and paired parallel membranes (Fig. 4*d*).

Infectivity of viral products produced in *N. rustica* protoplasts

To obtain further evidence that inoculation of protoplasts with TSWV resulted in newly synthesized infectious virus, petunia leaves were inoculated with samples of TSWV-inoculated *N. rustica* protoplasts. Protoplasts inoculated with TSWV and immediately applied to petunia leaves occasionally generated a few lesions (1 or 2 lesions per 10^6 protoplasts) due to the inoculum virus, but usually no lesions at all. In three independent experiments (using different batches of virus) the *N. rustica* protoplast suspensions harvested 40–64 h p.i. generated a clear increase in local lesion numbers (5 to 24 lesions per 10^6 protoplasts), with the infection percentages being relatively low in these experiments (30–40%).

Discussion

Development of a versatile analytical infection system, an obvious goal in research on tospoviruses, has not been reported before. Most likely a major problem has been, and still remains, the instability of the virus once isolated from the plant (Brunt *et al*., 1996). This problem can be partially circumvented by using a fast and efficient protocol for particle isolation, and by immediate use of the preparations. *In vitro*-grown *N. rustica* plants gave better and more reproducible results than greenhouse-grown plants, probably due to increased susceptibility of plants grown *in vitro*, and smaller seasonal effects.

The results presented in this paper show that a complete infection cycle occurs in protoplasts, and the characteristics of infection are very similar to what has previously been observed in infection of whole plants. The occurrence of tubular structures containing NSm on the surface of inoculated protoplasts, similar to those observed in protoplasts from pre-infected leaves (Storms *et al*., 1995), underscores the utility of the developed protoplast inoculation protocol for studying the tospoviral infection cycle. Additionally, by analogy with the expression of NSs, the synthesis of NSm is indicative of replication of TSWV input material.

TSWV infection in *N. rustica* protoplasts was more efficient than in *V. unguiculata* protoplasts. In particular, the production of viral glycoproteins, and subsequently enveloped particles, seemed hampered in *V. unguiculata* protoplasts, whereas the production of mature particles in *N. rustica* protoplasts was abundant. Electron microscopy on thin sections also showed that at whole plant level, the production of enveloped particles was hampered in *V. unguiculata* (data not shown). Apparently,

Fig. 4. Immuno-electron microscopical analysis of TSWV-inoculated *N. rustica* and *V. unguiculata* protoplasts. (*a*) Inoculum virus (V) adherent to *V. unguiculata* protoplasts at 0 h p.i. and labelled with anti-G1 serum; (*b*) viroplasm (VP) labelled with anti-N serum in *V. unguiculata* protoplasts at 40 h p.i.; (*c*) accumulation of nucleocapsid aggregates (NA) in *V. unguiculata* protoplasts at 52 h p.i. labelled with anti-N serum; (*d*) paired parallel membranes (PPM) surrounded by viroplasm in *N. rustica* protoplasts 40 h p.i. labelled with anti-N serum; (*e*) virus particles associated with and surrounded by endoplasmic reticulum membranes (arrowheads) in *N. rustica* protoplasts 40 h p.i. labelled with anti-G2 serum; (*f*) nucleocapsid aggregates and doubly enveloped virus (DEV) particles in inoculated *N. rustica* protoplasts 40 h p.i. labelled with anti-G1 serum. Bars represent 1 µm (A), or 0·5 µm (*b*, *c*, *d*, *e* and *f*).

V. unguiculata is a less suitable natural host than *N. rustica*, and our results indicate that the use of *N. rustica* protoplasts for studying TSWV infection is preferable.

The system is still relatively fragile, due to the instability of virus preparations and varying susceptibility of protoplasts, but it does make several interesting experiments possible. Specific agents that influence intracellular processes can now be applied, to gain insight into a number of aspects of the infection cycle. Among those are tunicamycin (which inhibits *N*-linked glycosylation) and Brefeldin A (which inhibits protein transport from the endoplasmic reticulum to the Golgi system in plants; Satiat-Jeunemaitre & Hawes, 1992*a*, *b*). The synchronism of the infection in the protoplast system also provides the potential to investigate the switch between transcription and replication that takes place during an infection. More detailed time-course experiments should pinpoint this switch, and by using protoplasts from N-protein-expressing transgenic plants (Gielen *et al*., 1991), the role of the level of nucleoprotein in inducing the switch can be investigated. The possibility of using protoplasts from transgenic plants also provides a tool for checking TSWV resistance at the cellular level. Experiments of this kind have recently been successfully performed in our laboratory by Prins *et al*. (1997).

Another obvious application for the developed system is the detailed investigation of TSWV particle maturation. Since the infection in protoplasts is far more synchronous than in whole plants, we may well be able to draw conclusions about the chronology of the events observed in infection. Immunofluorescence studies have given some preliminary indications concerning certain aspects of maturation. Both nucleocapsids and glycoproteins are found clustered in patches in late stages of infection (Fig. 3*b*, *d*), most probably depicting the accumulation of mature particles within (endoplasmic reticulum) membranes grouped within the cytoplasm (Fig. 4*e*). In the early stages, when few mature particles were formed, smaller patches were seen in immunofluorescence tests when looking at the glycoproteins, which might indicate that free glycoprotein tends to be concentrated at certain cellular sites. When using a marker for the plant Golgi apparatus, the pattern co-localized with the glycoproteins (data not shown). This leads to the suggestion that by analogy with the animal-infecting bunyaviruses, tospovirus glycoproteins accumulate in the Golgi system where possibly budding of TSWV particles also may take place. By exploiting the single-cell infection system described here, and by using detailed time-course analyses and specific inhibitors, we should be able to further unravel the maturation pathway of TSWV.

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