Expression of the tomato ringspot nepovirus movement and coat proteins in protoplasts

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Tomato ringspot virus (TomRSV), a bipartite plus-strand RNA virus, belongs to the nepovirus group. The complete nucleotide sequence of the TomRSV genome has recently been elucidated (Rott et al., 1991, 1995). TomRSV RNA-1 and RNA-2 encode polyproteins P1 and P2 respectively, which are subsequently cleaved by a virus-encoded protease to release the mature viral proteins. The genomic organization of TomRSV is similar to that of other nepoviruses. The C terminus of the RNA-2 polyprotein (P2) contains the domain for the movement protein followed by the domain for the coat protein. Activity of the RNA-1-encoded protease at the cleavage site between the movement protein and the coat protein has been characterized in vitro and in protoplasts (Hans & Sanfaçon, 1995). We previously obtained monoclonal antibodies against the movement protein which allowed us to detect a protein with an apparent molecular mass of 45 kDa in infected plants. This protein was found in association with tubular structures containing virus-like particles present in or near the cell wall (Wieczorek & Sanfaçon, 1993). This suggested a cell-to-cell movement function similar to that described for the comovirus and caulimovirus movement proteins (Shanks et al., 1989; Van Lent et al., 1990; Linstead et al., 1988) but different from that described for the tobacco mosaic virus (TMV) and most other plant virus movement proteins (for a review, see Maule, 1991). Most viral movement proteins (including the TMV movement protein) are present transiently in infected plants, i.e. at early stages of infection (for a review see Maule, 1991). In contrast, a caulimovirus movement protein was reported to be present at late stages of infection (Perbal et al., 1993). Expression patterns of viral movement proteins have most often been studied in whole plants, where infection of the different cells is not necessarily synchronous and where it is not possible to test directly for synthesis and stability of the proteins in relation to steady-state levels. In this study, we have examined the steady-state level, the production, and the stability of the TomRSV movement protein relative to the coat protein during the course of protoplast infection.

In order to detect the coat protein in immunoblots, polyclonal antibodies were raised against denatured viral particles. TomRSV particles were purified as described (Wieczorek & Sanfaçon, 1995), treated with 2 % SDS for 1 h at room temperature and dialysed extensively against PBS buffer. Rabbits were immunized with three intramuscular injections, 1 month apart, containing 100 μg of denatured antigen in incomplete Freund’s adjuvant. Blood was collected 2 weeks after the final injection. To test if the antiserum obtained could recognize the denatured coat protein, a preparation of purified virions was immunoblotted as described previously (Wieczorek & Sanfaçon, 1993). A band of apparent molecular mass 58 kDa was detected which co-migrated with the

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Coomassie blue stained band of purified denatured coat protein (result not shown).

A monoclonal antibody against the movement protein described previously (Wieczorek & Sanfaçon, 1993) and the polyclonal antiserum against the denatured coat protein, were then used to detect the mature movement and coat protein in protoplasts transfected with TomRSV viral RNA. In order to allow a direct comparison of their accumulation rate over time, the movement and coat proteins were detected simultaneously on a single immunoblot (Fig. 1). *Nicotiana plumbaginifolia* protoplasts (6 × 10^6) were transfected with 10 μg of purified viral RNA using polyethylene glycol as described previously (Wieczorek & Sanfaçon, 1995). Protoplasts were collected by centrifugation, resuspended in 50 μl of sample buffer (Bio-Rad) containing 10% SDS, and boiled for 5 min. The extracts were electrophoresed on denaturing SDS–polyacrylamide gel (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and viral proteins were immunodetected as described previously (Wieczorek & Sanfaçon, 1993) with the following modifications. Equivalent dilutions of a mouse monoclonal antibody raised against the movement protein and of rabbit polyclonal antibodies raised against the coat protein were used to detect simultaneously both coat protein and movement protein. The blot was further developed by sequentially first adding goat anti-rabbit antibodies conjugated to alkaline phosphatase and second rabbit anti-mouse antibodies also conjugated to alkaline phosphatase (BioCan Scientific) with a rigorous wash in PBS in between. Polyclonal antibodies against the denatured coat protein cross-reacted with a host protein of approximately 100 kDa that was present in both the virus-transfected protoplasts and in the control mock-inoculated protoplasts. However, two predominant proteins of apparent molecular masses 45 kDa and 58 kDa were detected in virus-transfected protoplasts but not in mock-inoculated protoplasts (Fig. 1). It was therefore concluded that these proteins were virus specific and corresponded to the movement protein and the coat protein respectively. This was confirmed by developing the blot with each antibody separately (results not shown). The movement protein specific antibody used either alone (not shown) or in combination with the coat protein antibodies (Fig. 1) allowed the detection of at least one other minor band of apparent molecular mass 42 kDa. This is consistent with our previous observation in infected plants where a doublet of bands of approximate apparent molecular masses 45 and 42 kDa was detected (Wieczorek & Sanfaçon, 1993). As the minor 42 kDa protein was present in a higher ratio relative to the predominant 45 kDa protein at a later time during infection of protoplasts, it may represent either degradation or further processing of the movement protein. Both movement and coat proteins were initially detected at 24 h post-infection and were still present at 72 h post-infection. However, the amount of coat protein increased slightly with time (up to 72 h) while the amount of movement protein decreased slightly with time (with a maximum at 24 h). This suggested that either the coat protein and the movement protein are expressed differentially or that they have different stabilities in the extractable fraction of the protoplast.

To test for the production of the two mature proteins, 2 × 10^6 protoplasts were transfected with 4 μg of purified viral RNA and labelled for 2 h by the addition of 25 μCi [35S]methionine directly to the culture medium at 24 h post-transfection. Immunoprecipitations with each antibody were performed as described previously (Hans & Sanfaçon, 1995). As expected, the monoclonal antibody against the movement protein and the polyclonal antibodies against the coat protein allowed the immunoprecipitation of proteins of apparent molecular masses 45 kDa and 58 kDa respectively (Fig. 2a). Once again at least one additional minor protein of apparent molecular mass 42 kDa was also immunoprecipitated with the movement protein antibodies. To allow a direct comparison of the rate of production of the movement and coat proteins over time, immunoprecipitations were also performed using both antibodies simultaneously (Fig. 2a). The relative ratio of each protein immunoprecipitated was similar to that obtained when immunoprecipitations were performed separately. We repeatedly found that the band corresponding to the movement protein was more intense than the band corresponding to...
the coat protein. This could be due in part to a greater affinity of the monoclonal antibody to the movement protein and/or to the Protein A beads relative to the affinity of the polyclonal antibodies to the coat protein and/or to the Protein A beads. In addition, the movement protein could be labelled to a higher specific activity than the coat protein due to the presence of eight methionines in the movement protein as compared to seven methionines in the coat protein. Time-course pulse-labelling experiments were repeated several times. To allow a more meaningful comparison of the steady-state levels and of the production of the proteins, the results shown in Fig. 2(b) were obtained from the same transfection experiments as the results shown in Fig. 1. Production of both mature proteins was initiated around 10 h post-infection at which point small amounts of labelled proteins could be immunoprecipitated (visible upon overexposure of the autoradiogram, data not shown), peaked at 23 to 47 h for both proteins, and was still detectable at 71 h post-infection although at a much lower level. The ratios of movement protein to coat protein immunoprecipitated at the different time points proved to be very similar when compared by scanning of the autoradiograms using a Bio-Rad video densitometer. These results suggested coordinate maturation of the coat and movement proteins from the newly synthesized precursor polyprotein. This processing appears to be very efficient in vivo as we were unable to detect precursors to either protein even with prolonged exposures of polyacrylamide gels containing in vivo labelled proteins immunoprecipitated at early or late time points in infection (Fig. 2 and data not shown).

Finally, to determine the fate of each protein in the extractable fraction of the protoplasts, pulse-chase experiments were performed (Fig. 3). Protoplasts were labelled for 2 h at 24 h post-infection as described above. Unlabelled methionine was then added to the medium to a final concentration of 2 mM and protoplasts were collected and processed by immunoprecipitation at various time points after the pulse (Fig. 3a). The coat protein was found to be very stable for up to 48 h after the pulse while the movement protein disappeared more quickly. Similar results were obtained with a comovirus movement protein (Rezelman et al., 1989). This result could be explained if the movement protein was either sequestered in an unextractable form in the protoplast or excreted into the culture medium. We have previously shown association of the TomRSV movement protein with tubular structures present in or near the cell wall in infected plants (Wieczorek & Sanfaçon, 1993). In addition, the movement protein segregates with the membrane and cell wall fractions and not with the cytosolic fraction upon fractionation of infected plant tissue (results not shown). Although the procedure we use for immunoprecipitation includes boiling the protoplasts in the presence of high levels of SDS, which should allow the solubilization of most membrane proteins, we cannot exclude the possibility that some of the movement protein is sequestered in an unextractable form. Tubular structures have been shown to be formed at the surface of protoplasts infected by comoviruses and caulimoviruses (Van Lent et al., 1991; Perbal et al., 1993). These tubules were found to be very fragile; they were broken off upon centrifugation of the protoplasts, and as a result the movement protein could be detected in the supernatant (Wellink et al., 1987). We have made several attempts to either immunoprecipitate the TomRSV movement protein from the supernatant (Fig. 3b) or to collect broken tubular structures by high-speed centrifugation of the supernatant as described by Perbal et al.
Fig. 3. Relative stability of the TomRSV movement and coat protein in infected protoplasts. Protoplasts were labelled at 24 h post-inoculation for 2 h (P) as in Fig. 2 and subsequently chased with cold methionine (2 mM) for the length of time (h) indicated above each lane. M is a mock inoculated control which was pulsed for 2 h at 24 h post-transfection. Immunoprecipitations were performed using a mixture of antibodies raised against the coat and the movement protein. Migration of the molecular mass markers is indicated on the left side of the gel and the expected migration for bands corresponding to the coat protein (CP) and the movement protein (MP) is indicated on the right side of the gel. (a) Analysis of the protoplast fraction. Protoplasts were collected and analysed as described in Fig. 2. (b) Analysis of the supernatant fraction. Following pelleting of the protoplasts, the supernatant fraction which corresponded to the culture medium was collected. Immunoprecipitations were performed as described in Fig. 2.

(1993) (results not shown). However, we could not detect the movement protein in those fractions. Additionally, we could not detect tubular structures at the surface of infected protoplasts by immunofluorescence using the monoclonal antibody against the movement protein. Therefore, either tubules are not formed in protoplasts infected by TomRSV or the epitope recognized by the antibody is not accessible in intact tubules using this technique.

Alternatively, the movement protein might be unstable in infected protoplasts and in the cytoplasmic fraction of infected plants. The movement protein might be either degraded or further processed at additional cleavage sites. The presence of the 42 kDa minor protein recognized by the movement protein antibody at a late time-point in the pulse-chase experiment with infected protoplasts (see Fig. 3a) is consistent with either possibility.

Although the amount of movement protein decreased slightly with time, considerable amounts of the movement protein could be detected at 72 h post-inoculation (see Fig. 1), a time at which viral protein synthesis has already decreased (see Fig. 2). In contrast, synthesis of the TMV movement protein and of its subgenomic RNA in infected protoplasts ceases by 8 h post-infection, while the coat protein is synthesized for up to 70 h post-infection (Blum et al., 1989; Watanabe et al., 1984). TMV and most other plant viruses are thought to move as a ribonucleoprotein complex where the viral RNA is directly associated with the movement protein (see Maule, 1991, for a review). Cell-to-cell movement could therefore occur before encapsidation of the virus takes place, allowing expression of the movement protein before coat protein accumulation. The presence of the TomRSV (this study) and of a caulimovirus (Perbal et al., 1993) movement proteins at late times during infection of protoplasts might be related to the mechanism of cell-to-cell movement of caulimo-, como- and nepoviruses. These viruses are thought to move as encapsidated particles through tubular structures composed at least in part of the movement protein. Although in protoplasts transfected with the cowpea mosaic virus movement protein, tubular structures can be formed in absence of viral particles (Wellink et al., 1993), examination of plant tissues infected by nepoviruses always show an association of tubular structures with virus-like particles (A. Wieczorek & H. Sanfaçon, unpublished results). As these tubules are seen traversing the cell wall or in the cytoplasm, one possibility is that the tubules assemble around virus particles in the cytoplasm. If this is the case, it would be facilitated by coordinate expression of the movement and coat proteins. The steady-state levels of the putative movement proteins from two other nepovirus have been reported to be transient in infected plants and it was thus concluded that the proteins were unstable (Demangeat et al., 1992; Hibrand et al., 1992). In contrast, the grapevine fanleaf nepovirus movement protein, which is present at late stages of infection of protoplasts and whole plants, is found mainly in the cytoplasmic fraction and was therefore suggested to be stable (Ritzenthaler et al., 1995). Expression and stability of these proteins have not
been studied in protoplasts and it is therefore difficult to compare these results with the results presented here.

Taken together, our results strongly suggest that the differences seen in the steady-state level of the movement and coat protein are due to the different stability of the proteins in the extractable fraction of the protoplasts and not to differential expression of the proteins. Since mature viral proteins are released from precursor polyproteins by proteolytic processing, processing at the cleavage site between the movement protein and the coat protein and at the cleavage site at the N terminus of the movement protein are probably not differentially regulated over time in vivo. We are currently analysing the cleavage site(s) at the N terminus of the movement protein in vitro.

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References


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