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Immunodetection of the proteins encoded by grapevine chrome mosaic nepovirus RNA2

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Fragments of the putative non-structural proteins (44K and 46K) encoded by RNA2 of grapevine chrome mosaic nepovirus (GCMV) were expressed as fusion proteins in *Escherichia coli* and used to raise specific antisera. All three proteins encoded by GCMV RNA2 (viral coat protein, and the 44K and 46K proteins) were detected by immunoblotting in subcellular fractions prepared from the leaves of infected *Chenopodium quinoa* plants, confirming a previously proposed model of the GCMV RNA2-encoded polyprotein. In addition to the 44K protein, one of the antisera detected a 90K protein presumably representing a precursor of the

44K and 46K proteins. Whereas the 44K and coat proteins could be detected in both soluble and membrane fractions, the 46K protein was found to be specific to the membrane fraction. Analysis of the kinetics of accumulation of the proteins showed that the 44K and 46K proteins were very transient whereas the coat protein was more stable and could be detected up to 21 days after inoculation. These results provide the first direct *in vivo* data supporting the maturation map of the GCMV RNA2 polyprotein deduced from *in vitro* experiments.

Introduction

The genome of Hungarian grapevine chrome mosaic nepovirus (GCMV) (Martelli & Quacquarelli, 1972) consists of two positive-sense ssRNAs of 7212 nucleotides (RNA1; Le Gall *et al.*, 1989) and 4441 nucleotides (RNA2; Brault *et al.*, 1989). These RNAs are polyadenylated at their 3' ends and, similarly to those of the closely related tomato black ring nepovirus (TBRV) (Mayo *et al.*, 1982), are probably covalently linked to a small protein (VPg) at their 5' end. The RNAs have the capacity to encode polyproteins of 250K (RNA1) (Le Gall *et al.*, 1989) and 146K (RNA2) (Brault *et al.*, 1989).

TBRV RNA1 can replicate independently of RNA2 in protoplasts, indicating that it encodes the viral proteins involved in replication (Robinson *et al.*, 1980). Indeed, the polyproteins encoded by RNA1 of different nepoviruses have the characteristic signature sequences of proteins involved in viral RNA replication (Greif *et al.*, 1988; Le Gall *et al.*, 1989; Ritzenthaler *et al.*, 1991). RNA1 also encodes a proteolytic activity responsible for the maturation of both RNA1- and RNA2-encoded polyproteins (Demangeat *et al.*, 1990, 1991). However, the presence of RNA2 is essential for infection of plants, indicating that this RNA encodes protein(s) involved in the spread of the virus in the plant. The N-terminal sequence of the GCMV coat protein has been determined (Brault *et al.*, 1989), demonstrating that this

protein maps to the C terminus of the 146K polyprotein encoded by RNA2.

Recently, Demangeat *et al.* (1991) have used *in vitro* translation experiments to study the processing of the polyproteins encoded by TBRV and GCMV RNA2. On the basis of their results they have proposed a processing model for both polyproteins. In the case of GCMV, after a primary cleavage freeing the coat protein from the C-terminal end, the remaining 90K precursor would be cleaved to yield 44K and 46K end products. Differential labelling experiments have indicated that the 44K product represents the N-terminal part of the polyprotein whereas the 46K product represents its internal part. In addition, their results demonstrate that the proteolytic activities encoded by GCMV and TBRV RNA1 are able to process the polyprotein encoded by GCMV RNA2 in a similar fashion, a fact which is not surprising because pseudorecombinants can be obtained between these two viruses (Doz *et al.*, 1980).

In this paper, we report the use of rabbit antisera against the GCMV coat protein and the 44K and 46K non-structural proteins to confirm the model of processing of the GCMV RNA2-encoded polyprotein. The antisera specific for the 44K and 46K proteins were raised using fusion proteins produced in *Escherichia coli*. All three mature proteins and the 90K precursor postulated from the *in vitro* model were detected by immunoblotting in subcellular fractions obtained from

infected *Chenopodium quinoa* leaves, providing for the first time direct *in vivo* support for the maturation model derived from *in vitro* data.

Methods

Virus propagation and purification. GCMV was obtained from Dr G. P. Martelli (University of Bari, Italy) and propagated under greenhouse conditions in *C. quinoa* or *C. murale*. Carborundum-dusted leaves were inoculated with infected plant tissue freshly homogenized in 50 mM-sodium phosphate buffer pH 7.4 containing 25 mM-ascorbic acid. Mock-inoculated controls were treated in the same way but using a healthy plant extract. The B and M particles and the corresponding RNAs were purified from *C. quinoa* leaves as described previously (Doz *et al.*, 1980).

Cloning of GCMV cDNAs in the expression vector. The expression vector pEX2 (Genofit; Stanley & Luzio, 1984) was used for expressing parts of the GCMV RNA2-encoded polyprotein as fusion proteins. This vector contains, under the control of the bacteriophage λ Pr promoter, a *cro*- β -galactosidase hybrid gene with a cloning linker at its 3' end. The host *E. coli* strain used was pop2136, which constitutively expresses a thermolabile repressor (cI_{ts857}) of the Pr promoter. This strain was transformed and grown as suggested by Genofit.

Plasmid p5'2 was obtained by inserting a blunt-ended GCMV RNA2 cDNA fragment (corresponding to positions 267 to 1119) into the *Sma*I site of pEX2. Plasmid pINT was obtained in a similar fashion, using a cDNA fragment corresponding to positions 1673 to 2529.

Expression and purification of fusion proteins. Induction of expression and purification of fusion proteins (in the form of insoluble cellular inclusions) were performed essentially as described by Kocken *et al.* (1988). The final pellet was resuspended in gel loading buffer (60 mM-Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 15% glycerol, bromophenol blue) and the fusion protein was further purified by preparative denaturing PAGE (Laemmli, 1970). After electrophoresis the protein was visualized by soaking the gel in ice-cold 1 M-KCl and excised with a razor blade. The gel pieces containing the fusion protein were stored at -20 °C before being used for immunization of rabbits.

Preparation of antisera. Polyacrylamide gel pieces containing approximately 1 mg of fusion protein were minced with a razor blade and the volume was adjusted to 500 μ l with sterile water. This suspension was emulsified with an equal volume of incomplete Freund's adjuvant before injection into rabbits. The rabbits ('Blanc du Bouscat') received six injections at 2 week intervals. Seven days after the last injection, the rabbits were bled, and the sera were collected and stored at -20 °C. Preimmune sera for use in control experiments were collected from all animals before immunization.

Subcellular fractionation. Two fractions enriched in different subcellular compartments were obtained essentially as described by Eggen *et al.* (1988) from inoculated or from apical, systemically infected *C. quinoa* or *C. murale* leaves. Fresh tissue (2 g) was ground with a Dounce homogenizer in 3 ml of buffer (50 mM-Tris-acetate pH 7.4, 10 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT and 0.5 mM-PMSF) and the homogenate was centrifuged for 15 min at 1000 g. The supernatant was adjusted to 20% glycerol and centrifuged at 30000 g for 30 min at 4 °C. The supernatant [soluble fraction (SF)] contained mainly the soluble proteins. The pellet was resuspended in 0.25 ml buffer (50 mM-Tris-acetate pH 8, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT, 0.5 mM-PMSF and 25% glycerol) and contained insoluble material, mainly membranes [cell membrane fraction (CMF)]. The samples were stored at -80 °C before electrophoresis.

Immunoblotting. After 10% SDS-PAGE (Laemmli, 1970) the proteins were transferred to nitrocellulose (BA 45, Schleicher & Schüll) according to the method of Towbin *et al.* (1979) (2 h at 600 mA). The nitrocellulose sheets were then preincubated in antiserum buffer [PBS (150 mM-NaCl, 1 mM-KH₂PO₄, 8 mM-Na₂HPO₄, 2 mM-KCl, pH 7.4) supplemented with 1% Tween 20, 5% non-fat dry milk] for 1 h at room temperature and then in the same buffer containing the antiserum at the appropriate dilution (1:200 for anti- β 5'2 and anti- β INT, 1:500 for anti-GCMV) for 3 h at room temperature. After three washes (20 min) in PBS containing 0.2% Triton X-100, 0.2% SDS, and two 10 min washes in PBS, the filters were incubated with goat anti-rabbit IgG coupled to peroxidase (Diagnostics Pasteur) diluted 1:1000 in antiserum buffer. After a series of washings as described above, the peroxidase reaction was carried out using 4-chloro-1-naphthol (Sigma) as the substrate as described by Hawkes *et al.* (1982).

Results

Characterization of antisera against GCMV 44K and 46K proteins

Two fragments of the polyprotein encoded by GCMV RNA2 were expressed in *E. coli* as fusions with the *cro*- β -galactosidase hybrid protein encoded by vector pEX2. As shown in Fig. 1, these two regions were selected to represent most of the putative 44K and 46K maturation products of the polyprotein (Demangeat *et al.*, 1991). Care was taken, when selecting these regions, to allow for some flexibility in the position of the cleavage site between these two products in order to avoid including sequences from one product in the fusion protein corresponding to the other one. The program ANTI-GEN (PC-GENE, Genofit) predicted that the major antigenic determinant of both fusion proteins is located in the viral part rather than in the 117K *cro*- β -galactosidase part. The two recombinant plasmids obtained as described in Methods (p5'2 expressing part of the 44K product and pINT expressing part of the 46K one) were introduced into *E. coli* strain pop2136. The fusion proteins were expressed after a temperature shift to 42 °C, which inactivates the cI repressor constitutively expressed by this strain. As expected, after induction *E. coli* cells harbouring plasmid p5'2 expressed a 154K fusion protein, whereas cells harbouring pINT expressed a 151K protein (result not shown).

Fusion proteins overproduced in *E. coli* were recovered as insoluble inclusions, further purified by preparative SDS-PAGE and used to immunize rabbits, yielding the anti- β 5'2 (p5'2 fusion protein) and anti- β INT (pINT fusion protein) antisera. In contrast to the preimmune sera, the antisera recognized both the cognate fusion protein and the *cro*- β -galactosidase hybrid protein in Western blotting experiments (result not shown). Their specificity was further demonstrated by immunoprecipitation experiments showing that the antisera against the



Fig. 1. Position on the GCMV RNA2-encoded 146K polyprotein of the parts expressed in *E. coli* as translational fusion proteins (shaded areas). The positions of the expressed parts are indicated by nucleotide numbers. The cleavage site of the coat protein is indicated by a vertical bar.

fusion proteins (but not the preimmune sera) immunoprecipitated the GCMV RNA2-encoded polyprotein (result not shown).

Detection and subcellular localization of viral protein in infected plants

Preliminary immunoblotting experiments, using total protein extracts from infected *C. quinoa* plants, did not allow the detection of any GCMV proteins other than the coat protein. In an attempt to enrich the preparations for various non-structural proteins, subcellular fractions from the infected plants were prepared. As described in Methods, these fractions were obtained according to the protocol of Eggen *et al.* (1988), which allows the separation of a SF and of a CMF. These fractions were prepared either from inoculated leaves or from the systemically infected apical leaves of the plants. According to data from preliminary experiments, fractions prepared 3 (44K and 46K proteins) and 5 days (coat protein) after inoculation were used. The proteins from the various fractions were then separated by 10% SDS-PAGE, transferred to nitrocellulose and subjected to immunodetection experiments using the anti- β 5'2, anti- β INT and anti-GCMV antisera. It should be noted that, for technical reasons, the SF and CMF analysed represent differing amounts of starting fresh plant material (32 mg per lane for SF, 320 mg per lane for CMF). Preimmune sera were used as controls throughout this study because cross-reactions between antisera prepared against fusion proteins and plant components have sometimes been observed (Niesbach-Klöggen *et al.*, 1990).

To be characterized as a viral protein in such experiments a protein must fulfil two criteria: it must be specifically recognized by the immune sera (as opposed to the preimmune control) and it must be present only among the proteins of GCMV-infected plants. Although some reactions were observed between the anti- β 5'2 and the anti- β INT preimmune sera controls and plant proteins, the preimmune sera did not recognize any protein specific to GCMV-infected tissues (not shown).

The anti- β 5'2 antiserum specifically recognized a 44K and a 90K protein in fractions from GCMV-infected

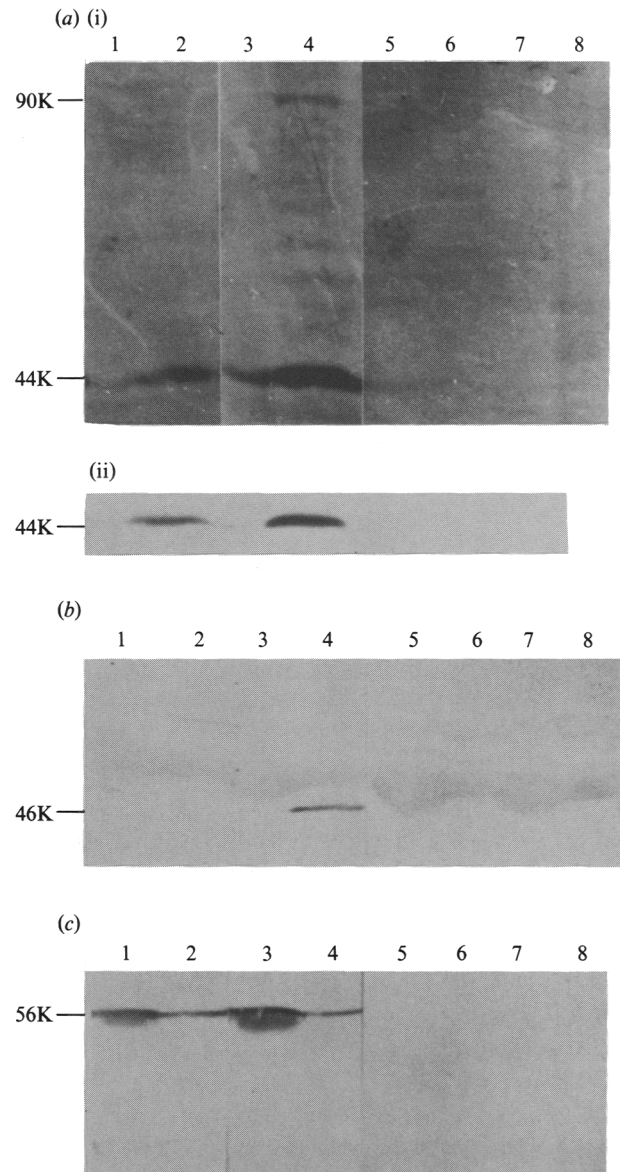


Fig. 2. Immunodetection of the proteins encoded by GCMV RNA2 in subcellular fractions prepared from GCMV-infected (lanes 1 to 4) or mock-inoculated (lanes 5 to 8) *C. quinoa* (a part i, b and c) or *C. murale* (a, part ii). The antisera used for detection were anti- β 5'2 (a), anti- β INT (b) and anti-GCMV (c). The fractions tested were the SF from apical leaves (lanes 1 and 5), CMF from apical leaves (lanes 2 and 6), SF from inoculated leaves (lanes 3 and 7) and CMF from inoculated leaves (lanes 4 and 8). The fractions analysed were prepared 3 days (a and b) and 5 days (c) after inoculation. The SF and CMF were equivalent to 32 mg and 320 mg of starting fresh plant material per lane.

plants (Fig. 2a, lanes 1 to 4). At the time of the experiment (3 days post-inoculation), the 44K protein was more abundant in the inoculated leaves than in the apical leaves. The 44K protein was detected in both the SF and CMF. The other protein detected, with an apparent M_r of 90K, was found specifically in the CMF of inoculated leaves (Fig. 2a, lane 4).

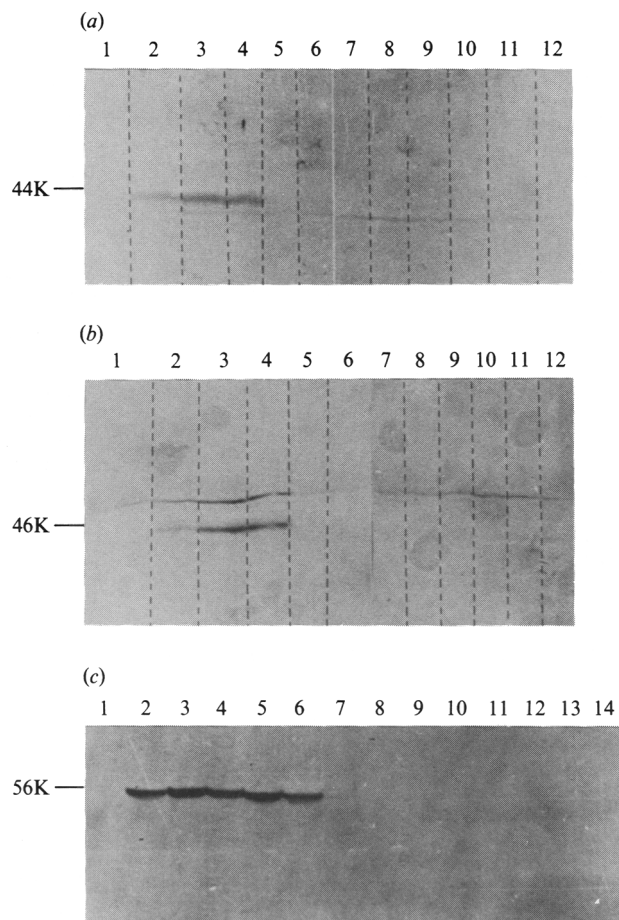


Fig. 3. Time course of accumulation of GCMV RNA2-encoded proteins in infected *C. quinoa* (*a* and *b*, lanes 1 to 6; *c*, lanes 1 to 7) and in control mock-inoculated (*a* and *b*, lanes 7 to 12; *c*, lanes 8 to 14) plants. In (*a*) and (*b*) samples were taken at 1 (lanes 1 and 7), 2.5 (lanes 2 and 8), 4 (lanes 3 and 9), 5 (lanes 4 and 10), 7 (lanes 5 and 11) and 10 (lanes 6 and 12) days post-infection; in (*c*) samples were taken at 1 (lanes 1 and 8), 3 (lanes 2 and 9), 5 (lanes 3 and 10), 6 (lanes 4 and 11), 7 (lanes 5 and 12), 11 (lanes 6 and 13) and 21 (lanes 7 and 14) days post-infection. Detection of (*a*) GCMV 44K protein and (*b*) 46K protein in the CMF from inoculated leaves using the anti- β 5'2 and the anti- β INT antiserum, respectively. (*c*) Detection of the coat protein in the SF from apical leaves using the anti-GCMV antiserum.

The anti- β INT antiserum detected a protein with an apparent M_r of 46K in the CMF from inoculated leaves of infected plants (Fig. 2*b*, lane 4). In *C. quinoa*, we were unable to detect this protein in any of the other fractions tested (SF, apical leaves, etc.). In another host of GCMV, *C. murale*, we were able to detect the 46K protein weakly in all the other fractions (not shown). Several other proteins reacted with the anti- β INT antiserum, but these proteins were also detected in fractions from mock-inoculated plants or when the preimmune serum was used, which shows that these proteins, unlike the 46K protein, are not encoded by GCMV.

As expected, the anti-GCMV antiserum detected the 56K coat protein, mainly in the SF from both apical and inoculated leaves (Fig. 2*c*, lanes 1 and 3). The coat protein was also detectable, although in lower amounts, in the CMF from apical and inoculated leaves.

Time course of accumulation of viral proteins

Using the fraction in which the various GCMV RNA2-encoded proteins were found to be most abundant in the experiments described above (CMF from inoculated leaves for the 44K and 46K proteins, SF from the apical leaves for the coat protein; Fig. 2*a* to *c*), the time course of the accumulation of these proteins was studied. Fractions prepared from mock-inoculated plants were used as controls.

The 44K and 46K proteins are detected only transiently in the CMF from inoculated leaves, from approximately 2.5 to 5 days post-inoculation (Fig. 3*a* and *b*). These two proteins accumulated to similar levels during this period, reaching their maximum level by day 4, slightly decreasing on day 5 and disappearing very rapidly after day 5 being undetectable by day 7. In other experiments using samples from slightly different times, a similar accumulation pattern was observed and these proteins were not detected 6 days post-inoculation (not shown).

On the other hand, the 56K coat protein showed a very different accumulation pattern. Although it was detected as early as the other two proteins, it did not begin to decrease in amount before day 7 and remained detectable (albeit faintly) up to 21 days post-inoculation (Fig. 3*c*). A similar pattern was also observed when analysing the accumulation of the coat protein in the SF from inoculated leaves (result not shown). These results obtained by Western blotting confirm previous data obtained using quantitative ELISA (unpublished results).

Discussion

GCMV RNA2 encodes a polyprotein of 146K which contains the coat protein from positions 811 to 1384 (Brault *et al.*, 1989). The model derived by Demangeat *et al.* (1991) from *in vitro* translation/maturation experiments predicts that the polyprotein matures in two steps: (i) cleavage of the C-terminal coat protein leaving a 90K precursor and (ii) cleavage of the 90K precursor to yield two mature products, an N-terminal 44K protein and an internal 46K protein. Antisera were prepared against these two putative non-structural proteins expressed as translational fusion proteins in *E. coli*.

The 46K protein of TBRV, which is the counterpart of the GCMV 46K protein, has previously been detected by Demangeat (1990) in infected plants and protoplasts. Our data demonstrate for the first time that all the proteins (44K, 46K, 90K and coat proteins) predicted by the RNA2-encoded polyprotein maturation model are indeed present in infected plants, providing strong direct support for the model.

In this study, the anti- β INT antiserum failed to detect the 90K protein. However, this may be explained in several ways, the most probable being the very low amounts of 90K protein found in infected plants and the low reactivity of the anti- β INT antiserum. This idea is supported by the fact that a very weak reaction between the anti- β INT antiserum and a protein of approximately 90K (not shown) was sometimes observed when *C. murale* was used as the GCMV host plant. The precise position of the cleavage site splitting the 90K precursor into the 44K and 46K proteins is still a matter of speculation. Another alternative, which cannot be discounted, is that the 90K protein might represent a dimer of the 44K protein. However, the highly denaturing sample buffer used for electrophoresis (10% SDS, 25% 2-mercaptoethanol, 150 mM-Tris-HCl pH 6.8) makes this hypothesis unlikely.

The data on the subcellular localization of these various proteins should be considered with care, especially in that, for technical reasons, the fractions analysed in the experiments reported in Fig. 2 represent different amounts of fresh plant material (32 mg for SF, 320 mg for CMF). Thus, for example the 44K protein signal is stronger with CMF, but on a plant weight basis it is likely that more 44K protein is present in the soluble form than is associated with membranes. Another limitation of this approach is, of course, that the type of subcellular fractionation used provides only relatively crude fractions frequently contaminated by other subcellular components. It is nonetheless noteworthy that the 46K protein, which has been suspected to represent the movement protein of nepoviruses on the basis of weak homologies with other such proteins (Meyer *et al.*, 1986, Brault *et al.*, 1989), has only been detected in the CMF fraction of *C. quinoa*. Demangeat (1990) has shown that the 46K protein of TBRV is found both in a cell membrane fraction comparable to our CMF and in a cell wall fraction, providing further support for the hypothesis that the 46K protein is the movement protein of the virus.

The time course of accumulation of the 46K and 44K proteins was investigated in the insoluble fraction of the inoculated leaves. The results show that these proteins are detected only transiently, approximately from 2.5 to 5 days post-inoculation. This contrasts with the accumulation of the coat protein, which is still detectable 21 days

post-inoculation, both in inoculated and in apical leaves. Because all three proteins are produced in equimolar amounts by the maturation of their polyprotein precursor, it is likely that this difference corresponds to a much lower stability of the non-structural proteins. Demangeat (1990) has obtained essentially similar results when comparing the accumulation of the 46K protein and the coat protein of TBRV. However, he was able to detect the 46K protein very faintly up to 11 days post-infection. This might reflect true differences between TBRV and GCMV or, alternatively, differences in the sensitivity of the respective detection systems.

The function of the 44K and 46K proteins is still a matter of debate. It is known that RNA2 of nepoviruses is necessary for cell-to-cell movement of the virus (Robinson *et al.*, 1980) and that it encodes the nematode vector specificity of the virus (Harrison *et al.*, 1974; Harrison & Murant, 1977). The very transient expression of the 44K and 46K proteins is contrary to the hypothesis that one of these proteins might be associated with nematode transmission of the virus (Brault *et al.*, 1989). A protein with such a function would be expected to be present whenever virus particles are available for transmission. Unless the accumulation patterns of these proteins are different in the roots of the infected plants, the results of this study might thus be taken as an indirect indication that the coat protein harbours all the determinants of the nematode transmission properties of nepoviruses. Based on weak sequence homologies, the 46K protein has been suspected to be the movement protein of the virus (Meyer *et al.*, 1986; Brault *et al.*, 1989; Koonin *et al.*, 1991). The subcellular localization and kinetics of accumulation of this protein are compatible with such a function, but do not provide any conclusive proof.

The 58K protein encoded by the M RNA of cowpea mosaic virus has been implicated in the replication of M RNA (Holness *et al.*, 1989; O. Le Gall, unpublished). By analogy, the 44K might have a similar function.

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