Detection of the movement protein of red clover necrotic mosaic virus in a cell wall fraction from infected *Nicotiana clevelandii* plants

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The movement protein of red clover necrotic mosaic virus (RCNMV) was expressed in *Escherichia coli* as a fusion with a maltose-binding protein using the vector pMAL-cRI and used to produce an antiserum. The RCNMV movement protein was detected in a cell wall fraction obtained from infected *Nicotiana clevelandii* leaf tissue by immunoblotting using the movement protein antiserum. The movement protein could be detected 6 h after inoculation and reached a maximum after 24 h. In contrast, the virus capsid protein, detected in a soluble fraction by immunoblotting using a capsid antiserum, continued to increase for 72 h after inoculation.

Red clover necrotic mosaic dianthovirus (RCNMV) has a genome of two positive-sense ssRNA components (Gould et al., 1981). RNA 1 (3.9 kb) has three open reading frames (ORFs) with the potential to encode (in the order 5' to 3') proteins of Mr 27000 (27K), 57K and 37K (Xiong & Lommel, 1989). The 57K protein has amino acid sequence motifs characteristic of nucleic acid polymerases (Argos, 1988; Xiong & Lommel, 1989) and helicases (Habili & Symons, 1989; Buck, 1990), suggesting a role in RNA replication; it may be expressed as an 88K fusion protein resulting from a ribosomal frameshift near the end of the ORF for the 27K protein (Xiong & Lommel, 1989). The capsid protein (Mr 37K to 40K) is translated from a subgenomic RNA derived from RNA 1 (Morris-Krsinich et al., 1983; Marriott & Buck, 1988; Osman & Buck, 1990). The proteins encoded by RNA 1 are consistent with its ability to replicate and produce virus particles in plant protoplasts in the absence of RNA 2 (Osman & Buck, 1987; Paje-Manalo & Lommel, 1989).

Production of lesions on inoculated leaves and systemic invasion of plants requires both RNA 1 and RNA 2 (Gould et al., 1981; Okuno et al., 1983; Osman et al., 1986), suggesting that RNA 2 encodes a protein required for cell-to-cell movement of the virus. RNA 2 (1.45 kb) contains a single ORF with the potential to encode a protein of Mr 35K to 36K (Lommel et al., 1988; Osman et al., 1991a), which has limited amino acid sequence homology with the movement proteins of brome mosaic virus (Lommel et al., 1988) and cowpea chlorotic mottle virus (Allison et al., 1989). Although a protein of 35K to 36K has been produced by *in vitro* translation of RNA 2 (Lommel et al., 1988; Xiong & Lommel, 1991; Osman et al., 1991b), this protein has not hitherto been detected *in vivo*. We now report the detection of the RCNMV movement protein in a cell wall fraction from infected *Nicotiana clevelandii* plants.

To produce an antiserum to the RCNMV movement protein, a fusion protein consisting of a maltose-binding protein fused to the whole of the movement protein (excluding the first methionine) was first produced in *Escherichia coli*. A full-length cDNA clone of RNA 2 of RCNMV Czech isolate TpM-34 in pUC19 (Osman et al., 1991b) was excised from the vector with *Hind*III and *Pst*I, and cloned into the corresponding sites of pEMBL9 (+). An *Eco*RI site was introduced at the start of the coding region of the movement protein by *in vitro* mutagenesis (Kunkel et al., 1987) using an oligonucleotide, AGGTAGGTTTGAATTCGCTATTC, which corresponds to nucleotides 66 to 88 of RNA 2 (Osman et al., 1991a). The coding region of the movement protein, together with the 3' untranslated region, was then excised with *Eco*RI and *Pst*I and cloned into pMAL-cRI (New England Biolabs), a derivative of pMAL-c (Maina et al., 1988), an expression vector designed to express proteins as fusions to the C-terminal end of the *E. coli* maltose-binding protein. At the fusion junction, the vector encodes the recognition sequence for Factor Xa protease, which allows the cleavage of the fusion protein into its two constituents.

*E. coli* DH5α cells containing the recombinant pMAL-cRI/RCNMV plasmid were grown to an OD₆₀₀₀ of 0.5 and expression of the fusion protein was induced with IPTG (0·3 mM). After disruption of the cells by sonication, the fusion protein was purified by binding to an amylose resin, followed by elution with maltose (Maina...
et al., 1988). In SDS–PAGE (Laemmli, 1970), followed by staining with Coomassie blue R-250, a band was detected in the approximate position expected for the fusion protein (predicted Mr, 78K), together with a slightly faster moving band (apparent Mr, 70K) and a doublet in the approximate position expected for the *E. coli* maltose-binding protein (Mr, 42K) (Fig. 1a, lane 1). After cleavage with Factor Xa protease, only two bands were detected, one in the position of the maltose-binding protein and the other which migrated with an apparent Mr of 36K as expected for the RCNMV movement protein (Fig. 1a, lane 2). The reason why the movement protein stained more intensely than the maltose-binding protein is not known. These two proteins were separated by chromatography on the amylose resin (Maina et al., 1988). The maltose-binding protein bound to the column and was eluted with maltose (Fig. 1a, lane 3), whereas the RCNMV movement protein did not bind to the column (Fig. 1a, lane 4).

An antiserum to the fusion protein was prepared in a rabbit by one intravenous injection [1 mg protein in 1 ml of 10 mM-sodium phosphate, 0.5 mM-NaCl, pH 7.2 (PBS buffer)] followed by four intramuscular injections (1 mg protein in PBS buffer, emulsified with an equal volume of Freund’s incomplete adjuvant) given at intervals of 4 weeks. The antiserum was mixed with an equal volume of glycerol and stored at -20 °C. A gel, similar to that shown in Fig. 1(a), was blotted onto Hybond-C membrane and probed with this antiserum (further diluted 1:200 in PBS). Proteins to which the antibodies had bound were then detected by incubation with Protein A–peroxidase and chloronaphthol (Sherwood, 1987) (Fig. 1b). It is clear that the antiserum contained antibodies both to the maltose-binding protein and to the RCNMV movement protein. The antiserum did not react with the virus capsid protein (not shown).

*N. clevelandii* seedlings were grown for 3 to 4 weeks after seed germination at 22 to 25 °C and then inoculated with sap from bean leaves infected with RCNMV-TpM-34. At various time intervals after inoculation, samples of the inoculated leaf tissue were processed either for movement protein detection or for coat protein detection (see below). For the former, a modification of the method of Albrecht et al. (1988) was used. One g leaf tissue was ground with a pestle and mortar in 1 ml ice-cold grinding buffer (GB; 10 mM-KCl, 5 mM-MgCl2, 0.4 mM-sucrose, 10% v/v glycerol, 10 mM-2-mercaptoethanol, 100 mM-Tris–HCl pH 7.5). The mixture was filtered through muslin and one-third volume of 200 mM-Tris–HCl buffer pH 6.8, containing 40 mM-dithiothreitol, 8% (w/v) SDS and 10% glycerol was added to the filtrate, followed by heating at 100 °C for 5 min, and centrifugation at 10000 g to remove insoluble material. This soluble fraction was designated S.

The material retained by the muslin was washed twice with GB buffer containing 2% (v/v) Triton X-100 by stirring and filtration again through muslin each time. The final residue was heated for 10 min at 100 °C with 0.2 ml SU buffer (4-5% SDS, 9 M-urea, 10 mM-2-mercaptoethanol, 75 mM-Tris–HCl pH 6.8) and then centrifuged at 10000 g to remove insoluble material. This cell wall extract was designated CW.

Fractions S and CW, from healthy leaf tissue or leaf tissue at different time intervals after inoculation, were subjected to SDS–PAGE, blotted onto Hybond-C membrane and probed with the movement protein antiserum. Proteins to which the antibodies had bound were then detected by incubation with Protein A–peroxidase and chloronaphthol (Sherwood, 1987). No protein was detected in either fraction from healthy leaf tissue, confirming the specificity of the antiserum. A protein with the same mobility as the movement protein was detected in the CW fraction from leaf tissue 6 h after inoculation, reached a maximum 24 h after inoculation and thereafter declined, although it could still be detected 96 h after inoculation (Fig. 2a). The movement protein, detected in the cell wall fractions, formed a closely spaced doublet in SDS–PAGE. Whether this indicates that there are two forms of the movement
the RCNMV capsid protein was also measured. Two-

protein after 24 h suggests that degradation of the

leaf tissue up to 96 h after infection. However, the

observed reduction.

fusion protein cleaved with Factor Xa protease. Lanes 2 to 9, cell wall

fractions from: healthy leaf tissue (lane 2); leaf tissue immediately after inoculation (lane 3); leaf tissue 6 (lane 4), 12 (lane 5),

24 (lane 6), 48 (lane 7), 72 (lane 8) and 96 h (lane 9) after inoculation. (b) Immunoblot using the capsid protein antiserum. Lanes 1 to 8, soluble

fractions from: healthy leaf tissue (lane 1); leaf tissue immediately after inoculation (lane 2); leaf tissue 6 (lane 3), 12 (lane 4), 24 (lane 5), 48

(lane 6), 72 (lane 7) and 96 H (lane 8) after inoculation. The positions of the maltose-binding protein (MBP), movement protein (MP) and
capsid protein (CP), and the $M_r$ values of protein standards (as in Fig.
1) are shown.

protein will require further investigation. It is noteworthy that the movement protein produced in E.
coli, using the pMAL-cRI vector, also formed a closely
spaced doublet. The decline in the level of the movement protein after 24 h suggests that degradation of the movement protein had occurred, since expansion of the
N. clevelandii leaves was insufficient to account for the observed reduction.

No movement protein was detected in fraction S from
leaf tissue up to 96 h after infection. However, the
possibility that some movement protein could have been
present in a membrane subfraction of fraction S (pelletable at 30000 $g$; Pe-30) cannot be discounted,
since pelleting this subfraction would have concentrated
the movement protein (if present) and hence increased
the sensitivity of detection. It is noteworthy that some of
the putative movement protein of alfalfa mosaic virus
was located in Pe-30, although the majority was found in
the cell wall fraction (Godefroy-Colburn et al., 1986).

For comparison, the time course of accumulation of
the RCNMV capsid protein was also measured. Two-
hundred mg leaf samples were ground in 50 mM-Tris-
HCl, 2% SDS, 10 mM-dithiothreitol, 10% glycerol,
pH 6-8, heated at 100 °C for 5 min, cooled, centrifuged to
remove insoluble debris and subjected to SDS–PAGE.
After electrophoresis, the gel was blotted onto Hybond-C
membrane and the blot was incubated with an anti-

erum to the RCNMV capsid protein (diluted 1 : 1000 in
PBS) (Osman & Buck, 1987). Proteins to which antibodies had bound were detected as before (Sher-
wood, 1987). The amount of coat protein detected
increased with time reaching a maximum 72 h after
inoculation.

There is accumulating evidence that cell-to-cell move-
ment of plant viruses occurs through intercellular
connections, the plasmodesmata, and that such move-
ment is mediated by virus-encoded movement proteins
(Hull, 1989; Robards & Lucas, 1990; Atabekov &
Taliiansky, 1990). Immunocytological studies have
shown that the movement protein of tobacco mosaic
virus (TMV) is localized in the plasmodesmata, both in
infected plants and in uninfected transgenic plants
expressing the movement protein from a chromosomally
inserted gene (Tomenius et al., 1987; Atkins et al., 1991),
and that the protein is found in a cell wall-enriched
fraction after subcellular fractionation of tissue from
infected plants (Moser et al., 1988; Lehto et al., 1990).

Putative movement proteins of a number of other
viruses, e.g. alfalfa mosaic virus (Godefroy-Colburn et
al., 1986; Stussi-Garaud et al., 1987) and cauliflower
mosaic virus (Albrecht et al., 1988; Linstead et al., 1988)
have also been localized to the cell wall or plasmodes-
mata. The finding of the RCNMV movement protein
in a cell wall fraction therefore is consistent with the
possibility that this virus also moves between cells via the
plasmodesmata. However, immunocytological studies
will be needed to determine the subcellular location of its
movement protein more precisely.

The finding that the RCNMV movement protein
reaches a maximum 24 h after inoculation, whereas the
capsid protein continues to accumulate up to 72 h is
consistent with similar studies of the accumulation of the
TMV movement protein (Lehto et al., 1990) which
indicated that most of the movement protein accumulat-
ed during the early phase of virus replication. If only
single-stranded nucleic acids complexed with viral
movement proteins can be transported efficiently
through plasmodesmata, as speculated recently by
Citovsky et al. (1991), a temporal separation of move-
ment protein and capsid protein production would
reduce competition for binding to the same RNA.
Further studies will be needed to determine whether the
RCNMV movement protein binds to single-stranded
nucleic acids.

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References


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