Replication of Red Clover Necrotic Mosaic Virus RNA in Cowpea Protoplasts: RNA 1 Replicates Independently of RNA 2

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SUMMARY

Inoculation of cowpea mesophyll protoplasts with unseparated RNA 1 and RNA 2 from red clover necrotic mosaic virus in the presence of polyethylene glycol resulted in virus RNA replication, the synthesis of virus capsid polypeptide and the formation of virus particles; 75 to 85% of the viable protoplasts became infected and the yield of virus particles or virus RNA after 72 h incubation corresponded to about $3 \times 10^6$ virus genomes per infected protoplast. In contrast, no replication could be detected when protoplasts were inoculated with RNA 2 alone. However, inoculation of protoplasts with RNA 1 alone resulted in its replication and the formation of virus particles, with a yield similar to that obtained after inoculation with both RNAs. Since infection of plants requires both RNA 1 and RNA 2 to be present, the demonstration of the independent replication of RNA 1 in single cells strengthens the hypothesis that RNA 2 plays a role in the cell-to-cell transmission of the virus.

INTRODUCTION

Red clover necrotic mosaic virus (RCNMV) belongs to the dianthovirus group (Matthews, 1982) members of which are characterized by having isometric particles approx. 30 nm in diameter, two single-stranded RNA species, RNA 1 (approx. 4500 nucleotides) and RNA 2 (approx. 1500 nucleotides), both of which are required for plants to become infected, and a single capsid polypeptide species, mol. wt. 40000, encoded by RNA 1 (Hollings & Stone, 1977; Dodds et al., 1977; Gould et al., 1981; Okuno et al., 1983; Morris-Krsinich et al., 1983). The properties of pseudorecombinants formed between RCNMV and two other dianthoviruses, sweet clover necrotic mosaic virus and clover primary leaf necrosis virus, indicate that symptom type in several plant species is determined by RNA 1 or by interaction between RNA 1 and RNA 2 (Okuno et al., 1983). However, similar studies show that, for two strains of RCNMV (Czechoslovakian strain TpM-34 and English strain H), the morphology of lesions in cowpea and the ability to invade the plants systemically is determined primarily by RNA 2 (Osman et al., 1986).

If the major function of RNA 2 is to promote cell-to-cell spread of the virus, it seemed possible that RNA 1 could replicate on its own in single cells. There have been no previous reports on the replication of any dianthovirus in isolated protoplasts. In this paper we report infection of cowpea protoplasts following inoculation with unseparated RCNMV RNA 1 and RNA 2, and the results of inoculation with RNA 1 and RNA 2 individually.

METHODS

Preparation of virus and viral RNA. RCNMV strain TpM-34 (Musil, 1969; Hollings & Stone, 1977), which had been subjected to three cycles of single lesion isolation in Chenopodium quinoa (Osman et al., 1986), was propagated in Phaseolus vulgaris cv. 'The Prince' (Hollings & Stone, 1977) and extracted and purified by differential and sucrose density gradient centrifugation (Gould et al., 1981). For use in antiserum production, virus was further purified by centrifugation through linear gradients of 10 to 86% (w/v) caesium chloride in 10 mM-sodium phosphate buffer pH 7.0, at 25000 r.p.m. in a Beckman SW28 rotor for 16 h at 4 °C. The virus particles, which
banded at a density of 1.36 g/ml (Hollings & Stone, 1977), were collected and dialysed against 10 mM-sodium phosphate buffer pH 7.0. RNA was isolated from virus particles by phenol/SDS extraction (Okuno et al., 1983). The two RNA species were separated by three cycles of centrifugation in sucrose/formamide gradients (Osman et al., 1986). RNA was stored at −70 °C in sterile distilled water.

**Preparation of virus antiserum and IgG.** A rabbit was injected intravenously with purified RCNMV (2 mg), followed 1 and 3 weeks later by two intramuscular injections with equal amounts of virus emulsified with Freund's complete adjuvant. Antiserum was prepared from blood collected 3 weeks after the last injection and stored at −20 °C after the addition of an equal volume of glycerol. The IgG fraction of the antiserum was prepared as described by Clark & Adams (1977).

**Protoplast isolation and infection.** Protoplasts were obtained from cotyledons of 10- to 12-day-old cowpea plants [Vigna unguiculata (= V. sinensis) cv. California Blackeye No. 5] cultivated in a growth room [16 h light (10000 lux), 25 °C; 8 h dark, 22 °C] in vermiculite and watered with a solution of Heller's (1953) salts. After the lower epidermis had been detached, leaves were floated on a solution containing 0.5% cellulose ‘Onozuka’ R-10, 0.1% macerozyme R-10 and 0.1% bovine serum albumin in 0.5 M mannitol, pH 5.5 and incubated at 30 °C for 3-5 h on a rotary shaker (25 cycles/min). The released protoplasts were then pelleted by centrifugation at 42 g for 1 min. Inoculation of protoplasts with viral RNA was essentially as described by Maule et al. (1980). A pellet containing 4 × 10⁶ to 5 × 10⁶ protoplasts was mixed with a solution (2 ml) containing 40% (w/v) polyethylene glycol (PEG) (mol. wt. 6000), 3 mM CaCl₂, and viral RNA (11 μg RNA 1 or 11 μg RNA 2 or 11 μg of each RNA) and, after 10 s, the suspension was diluted with 0.5 M mannitol, 0.1 mM CaCl₂ (20 ml). Mock inoculations were the same except that virus RNA was omitted. After 30 min at room temperature, the protoplasts were pelleted by centrifugation (42 g, 1 min), washed three times in 0.5 M mannitol, 0.1 mM CaCl₂, and resuspended in 6 ml of the incubation medium described by Aoki & Takebe (1969), containing carbenicillin (200 μg/ml) and nystatin (75 units/ml). The protoplasts were kept at 22 °C in continuous light at 3000 lux for 72 h. Their viability was determined by staining with phenosafranine (Widholm, 1972). For investigation of virus protein synthesis [35S]methionine (10 μCi/ml) and chloramphenicol (200 μg/ml) were added immediately after inoculation, followed 15 h later by actinomycin D (10 μg/ml). Samples of protoplast suspension (1 ml) were withdrawn at intervals and the protoplasts were counted, pelleted and stored at −70 °C.

**Infectivity assays.** Thawed protoplast pellets were ground in 0.1 M-sodium phosphate buffer, pH 7.0 (500 μl) and the suspension was inoculated to carborundum-dusted cowpea plants (10 to 12 days old, two leaves per plant). Lesions were counted six days after inoculation.

**ELISA.** Thawed protoplast pellets were ground in 0.02 M-sodium phosphate, 0.15 M NaCl, 0.05% Tween 20, pH 7.4 (500 μl) and, after centrifugation (3000 g, 10 min) to remove debris, viral antigen was assayed by ELISA (Clark & Adams, 1977) using anti-RCNMV IgG coupled to alkaline phosphatase.

**Fluorescent antibody staining of protoplasts.** The indirect fluorescent antibody method described by Wood (1985) was used; anti-RCNMV serum was diluted 1:32 and fluorescein-labelled sheep anti-rabbit immunoglobulin (Wellcome) was diluted 1:16.

**Immunosorbent electron microscopy (ISEM).** Thawed protoplast pellets were ground in 0.1 M-sodium phosphate buffer pH 7.0 (200 μl) and, after centrifugation (3000 g, 10 min) to remove debris, particles of RCNMV were detected by ISEM as described by Roberts & Harrison (1979), using a 1:500 dilution of anti-RCNMV serum and formalin-fixed Staphylococcus aureus cells (Immunoprecipitin, Bethesda Research Laboratories) as described by Arbison et al. (1985). Immunoprecipitates were suspended in sample buffer (Laemmli, 1970) and heated at 100 °C for 3 min prior to SDS–PAGE.

**SDS–PAGE of proteins.** Thawed pellets of [35S]methionine-incubated protoplasts were ground in 10 mM-KCl, 2 mM-EDTA, 5 mM-2-mercaptoethanol, 50 mM-Tris-HCl pH 7-6 (50 μl). After centrifugation (3000 g, 10 min) to remove debris, the suspension was mixed with an equal volume of sample buffer (Laemmli, 1970), heated to 100 °C for 3 min and subjected to SDS–PAGE in a 3% (w/v) stacking gel and a 10% (w/v) separating gel (Laemmli, 1970). Gels were stained with 0.2% Coomassie Brilliant Blue R250 in 30% (v/v) methanol, 7% (v/v) acetic acid, destained in the solvent and then soaked in distilled water followed by 1 M-sodium salicylate (Chamberlain, 1979), prior to fluorography.

**Immunoprecipitation of 35S-labelled capsid polypeptide.** Extracts of protoplasts were mixed with anti-RCNMV IgG and formalin-fixed Staphylococcus aureus cells (Immunoprecipitin, Bethesda Research Laboratories) as described by Arbison et al. (1985). Immunoprecipitates were suspended in sample buffer (Laemmli, 1970) and heated at 100 °C for 3 min prior to SDS–PAGE.

**Dot hybridization and Northern blotting.** Thawed protoplast pellets were mixed into 50 mM-Tris–HCl pH 7-5, 5 mM-EDTA, 100 mM-NaCl, 1% SDS (0-2 ml) and extracted three times with water-saturated phenol and once with chloroform : isooamyl alcohol (24:1 by vol.). Nucleic acids were precipitated from the aqueous phase with ethanol, resuspended in sterile distilled water and denatured by heating at 65 °C for 5 min in 50% (v/v) formamide (deionized), 6-4% (w/v) formaldehyde, 5 mM-sodium acetate, 1 mM-EDTA, 20 mM-sodium MOPS pH 7.0. Samples were then either spotted directly onto GeneScreen Plus membrane, or electrophoresed in a 1% agarose gel containing 6-4% (w/v) formaldehyde, 5 mM-sodium acetate, 1 mM-EDTA, 20 mM-sodium MOPS pH 7.0, transferred to GeneScreen Plus membrane by capillary blotting and then hybridized with [32P]-labelled probes in
Replication of RCNMV RNA in protoplasts

50% (v/v) formamide, according to the protocol recommended by the manufacturer (New England Nuclear). $^{32}$P-labelled cDNA probes to RCNMV RNA 1 and RNA 2 were prepared as described by Osman et al. (1986). $^{32}$P-labelled probes from plasmids pBM3 and pGC9 were prepared by nick translation (Rigby et al., 1977). Plasmids pBM3 and pGC9 contained 520 bp and 355 bp double-stranded cDNAs prepared to RCNMV RNA 1 and RNA 2 respectively, cloned into the $PstI$ site of pUC8 (A. C. Marriott & K. W. Buck, unpublished results) and were kindly donated by A. C. Marriott.

RESULTS

Infection of cowpea protoplasts with unseparated RCNMV RNA 1 and RNA 2

After inoculation of cowpea protoplasts with unseparated RCNMV RNA 1 and RNA 2 in the presence of PEG and incubation for 72 h, suspensions contained $3 \times 10^5$ to $5 \times 10^5$ viable protoplasts per ml of which 75 to 85% had synthesized virus capsid antigen as indicated by the indirect fluorescent antibody test using anti-RCNMV immunoglobulin. No fluorescence was observed in mock-inoculated protoplasts incubated for the same time period.

Evidence for infection of the protoplasts was also obtained by the increase in infectivity of protoplast extracts, measured by local lesion assay on cowpea and the synthesis of capsid polypeptide antigen, assayed by ELISA, over the 72 h incubation period (Table 1). Assuming that RCNMV particles contain 20% RNA (Hollings & Stone, 1977) and that all capsid antigen synthesized is converted into virions, the amount of capsid antigen synthesized after 72 h corresponds to 9 pg virus RNA per infected protoplast; assuming equal molar proportions of the RNA species and that each is a genome part, this is equivalent to $2.8 \times 10^6$ virus genomes per infected protoplast.

To determine the size of the virus capsid antigen synthesized, protoplasts inoculated with RCNMV RNA 1 and RNA 2 and mock-inoculated protoplasts were incubated in the presence of $[^{35}S]$methionine and protoplast extracts were immunoprecipitated with anti-RCNMV immunoglobulin and formalin-fixed S. aureus cells. Analysis of the immunoprecipitates by SDS-PAGE (Fig. 1) showed that a protein which comigrated with coat protein was specifically immunoprecipitated from the infected protoplast extracts, but no such protein could be detected after immunoprecipitation of extracts of mock-infected protoplasts. ISEM showed that inoculated but not mock-inoculated protoplasts contained numerous isometric particles indistinguishable from particles of RCNMV.

Nucleic acid samples from protoplasts at various times after inoculation with RNA 1 and RNA 2 were spotted onto GeneScreen membrane and probed with $^{32}$P-labelled cDNA prepared to RNA 1 and RNA 2. The radioactive areas were cut from the membrane, assayed by scintillation counting and compared with spots of known quantities of purified viral RNA. The accumulation of viral RNA paralleled the increase of infectivity and the production of capsid polypeptide antigen (Table 1); after 72 h infected protoplasts contained approximately $3.4 \times 10^6$ genome equivalents per infected protoplast. The similarity of this value to that determined by ELISA suggests that most of the RNA produced was encapsidated. A Northern blot of RNA from infected protoplasts (Fig. 2) showed that the proportions of RNA 1 and RNA 2 detected at different times during the incubation period were similar.

Independent replication of RCNMV RNA 1 in cowpea protoplasts

Separation of RCNMV RNA by three cycles of centrifugation in formamide–sucrose density gradients resulted in preparations of RNA 1 and RNA 2 which appeared homogeneous by formaldehyde–agarose electrophoresis followed by toluidine blue staining (result not shown). No cross-contamination could be detected when preparations of separated and unseparated RNA were electrophoresed in a formaldehyde–agarose gel, transferred to GeneScreen Plus membrane and probed with $^{32}$P-labelled cDNA probe to unseparated RNA (Fig. 3a). When a similar Northern blot was probed with $^{32}$P-labelled pGC9 (Fig. 3b), RNA 2 was detected in lane 1 (unseparated RNA) and lane 3 (RNA 2) but not in lane 2 (RNA 1), confirming the absence of RNA 2 in the RNA 1 preparation. Infectivity assays confirmed the purity of the RNA preparations because no lesions were obtained on any of the plants 6 days after inoculation with
Table 1. Assays of infectivity, capsid antigen and virus RNA in protoplasts inoculated with RNA 1 and RNA 2*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Infectivity†</th>
<th>Capsid antigen (pg)‡</th>
<th>Virus RNA (pg)‡</th>
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<tr>
<td>0</td>
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<tr>
<td>72</td>
<td>40</td>
<td>45</td>
<td>11</td>
</tr>
<tr>
<td>Mock-inoculated (72 h)</td>
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<td>0</td>
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* Infectivity, capsid antigen and virus RNA were assayed in separate experiments. Values are averages of two determinations; individual values differed from the average by less than 15%.
† Total number of lesions on two cowpea leaves, 6 days after inoculation with protoplast extracts.
‡ Amounts per infected protoplast.

Fig. 1. Synthesis of coat protein in protoplasts inoculated with unseparated RCNMV RNA 1 and RNA 2. Proteins were labelled with [35S]methionine, immunoprecipitated with anti-RCNMV IgG and detected by fluorography. Lanes 1 and 3, mock-inoculated protoplasts after 24 h and 48 h incubation. Lanes 2 and 4, inoculated protoplasts after 24 h and 48 h incubation. CP marks the position of RCNMV coat protein included as an internal standard.

Fig. 2. Northern blot of RCNMV RNA in extracts of protoplasts inoculated with unseparated RCNMV RNA. Lane 1, mock-inoculated protoplasts after 72 h incubation. Lanes 2 to 5, inoculated protoplasts after 0 h, 24 h, 48 h and 72 h incubation. Lane 6, RCNMV RNA. The probe was 32P-labelled cDNA to RCNMV RNA 1 and RNA 2.
Fig. 3. Northern blots of separated RCNMV RNA species. Lane 1, unseparated RCNMV RNA; lane 2, RNA 1; lane 3, RNA 2. The probes were (a) $^{32}$P-labelled cDNA to RCNMV RNA 1 and RNA 2; (b) $^{32}$P-labelled, nick-translated pGC9.

Fig. 4. Northern blot of RCNMV RNA in extracts of protoplasts inoculated separately with RNA 1 and RNA 2. Lane 1, mock-inoculated protoplasts after 72 h incubation. Lanes 2 to 5, protoplasts inoculated with RNA 1 after 0 h, 24 h, 48 h and 72 h incubation. Lane 6, RCNMV RNA. Lanes 7 to 10, protoplasts inoculated with RNA 2 after 0 h, 24 h, 48 h and 72 h incubation. The probe was $^{32}$P-labelled cDNA to RCNMV RNA 1 and RNA 2.

RNA 1 or RNA 2, whereas an average of 42 lesions per leaf was induced in leaves inoculated with the same concentration of unseparated RNA.

Batches of protoplasts were inoculated separately with preparations of RNA 1 and RNA 2 and incubated for 72 h. Nucleic acids, extracted from the protoplasts at various time intervals after inoculation, were separated by formaldehyde–agarose gel electrophoresis, transferred to GeneScreen Plus membrane and probed with $^{32}$P-labelled cDNA prepared to a mixture of RNA 1 and RNA 2 (Fig. 4). In both cases small amounts of residual input RNA could be detected immediately after inoculation (lanes 2 and 7). However, whereas the amount of RNA 1
increased with time (lanes 3 to 5), RNA 2 decreased in amount and could not be detected after 24 h and later (lanes 8 to 10). It was concluded that RNA replication occurred in protoplasts inoculated with RNA 1, but not in those inoculated with RNA 2.

In protoplasts inoculated with RNA 1, a small amount of an RNA with a mobility similar to that of RNA 2 was detected after 72 h (Fig. 4, lane 5). In order to test whether this was a subgenomic RNA derived from RNA 1 or the result of replication of previously undetected traces of RNA 2 in the inoculum, two experiments were carried out. Firstly, extracts of $2 \times 10^5$ protoplasts, 48 h and 72 h after inoculation with RNA 1, were assayed for infectivity and, unlike extracts of protoplasts inoculated with both RNAs (Table 1), induced no lesions on cowpea leaves. Secondly, Northern blots of nucleic acid samples extracted from protoplasts at various times after inoculation with RNA 1 were first probed with $^{32}$P-labelled pGC9 (Fig. 5a) which detected only the band of RNA 2 in the control sample of unseparated RCNMV RNA (lane 6); no bands in the position of RNA 2 could be detected in any of the inoculated protoplast extracts (lanes 2 to 5) after 5 days autoradiography. The same blot was then probed with $^{32}$P-labelled pBM3 and autoradiographed for 5 days (Fig. 5b), which showed that RNA 1 had accumulated over the 72 h period, together with a small amount of an RNA with a mobility similar to that of RNA 2. Since this smaller RNA hybridized with the RNA 1-specific probe, but not with the RNA 2-specific probe, it must have derived from RNA 1.

In order to determine whether virus capsid protein and particles were synthesized in protoplasts inoculated with the separate RNA species, extracts of inoculated protoplasts were assayed by ELISA and ISEM. In protoplasts inoculated with RNA 1 the amount of capsid antigen accumulation, assayed by ELISA, over the incubation period was similar to that in protoplasts inoculated with both RNAs (Table 1) reaching a maximum of 40 pg per viable protoplast after 72 h. The production of isometric particles with the same diameter as virions of RCNMV in such protoplasts was shown by ISEM. In contrast no synthesis of capsid antigen or
production of virus particles could be detected over the same incubation period either in mock-
inoculated protoplasts or in protoplasts inoculated with RNA 2.

**DISCUSSION**

The amount of RNA and capsid antigen synthesized in protoplasts inoculated with RCNMV RNA, which corresponds to about 3 × 10^6 genome equivalents per infected protoplast, is at the upper end of the range of 0.5 × 10^6 to 10 × 10^6 virions or genome equivalents per protoplast reported for a range of viruses in protoplasts of barley, *Chenopodium quinoa*, cowpea, tobacco or turnip (Mühlbach, 1982; De Varennes *et al.*, 1984; Harbison *et al.*, 1985). Such yields, combined with the ability routinely to infect 75 to 85% of the viable protoplasts makes the RCNMV/cowpea protoplast system described here attractive for studies of the replication of RCNMV and possibly of other dianthoviruses.

The independent replication of RNA 1 in protoplasts, combined with the synthesis of capsid protein and the formation of virus particles, but the inability of RNA 1 alone to invade plants systemically supports the hypothesis, based on the properties of pseudorecombinants formed between systemic and non-systemic strains of RCNMV on cowpea (Osman *et al.*, 1986), that RNA 2 is required for cell-to-cell transmission of the virus. RNA 2 programmes the synthesis in reticulocyte lysate of a protein of mol. wt. 34000 (Morris-Krsinich *et al.*, 1983; A. C. Marriott & K. W. Buck, unpublished results). Several RNA-containing plant viruses encode proteins of mol. wt. 30000 to 35000 which are thought to play a role in the cell-to-cell movement of the virus (Davies & Hull, 1982; Zimmern & Hunter, 1983). However, RCNMV may offer an advantage over other viruses for the study of its transport protein, since this is encoded by a separate RNA and hence can be studied independently of RNA replication or synthesis of coat protein.

The synthesis of coat protein in protoplasts inoculated with RNA 1 confirms the previous result, based on the properties of pseudorecombinants that RNA 1 encodes the coat protein (Okuno *et al.*, 1983; Osman *et al.*, 1986). From the results of *in vitro* translation, Morris-Krsinich *et al.* (1983) suggested that RCNMV coat protein may be translated from a subgenomic species of RNA 1 and it has recently been shown for strain TpM-34 that this subgenomic RNA has an electrophoretic mobility similar to that of RNA 2 (A. C. Marriott & K. W. Buck, unpublished results). The smaller of the two RNAs detected in protoplasts inoculated with RNA 1 has been shown to be a subgenomic RNA derived from RNA 1 and is probably the messenger RNA for coat protein.

Independent replication of RNA 1 in protoplasts has been reported for a number of viruses with bipartite genomes from other virus groups, i.e. cowpea mosaic comovirus (Goldbach *et al.*, 1980), tomato black ring nepovirus (Robinson *et al.*, 1980) and tobacco rattle tobravirus (Lister & Bracker, 1969; Sänger, 1969). However, unlike RCNMV and other dianthoviruses the coat proteins of all these viruses are encoded by RNA 2 and hence no particles are formed in protoplasts inoculated with RNA 1 alone. In protoplasts inoculated with RCNMV RNA 1, virus particles were formed and the amount of capsid protein produced was similar to that in protoplasts inoculated with both RNA species. Hence at the single cell level RNA 1 appears to contain all the information required for a complete virus replication cycle.

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**REFERENCES**


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