Independent Replication of Red Clover Necrotic Mosaic Virus RNA-1 in Electroporated Host and Nonhost *Nicotiana* Species Protoplasts

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ABSTRACT

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The two nonhomologous genomic single-stranded RNA components (RNA-1 and -2) of red clover necrotic mosaic virus (RCNMV) failed to elicit systemic symptoms when mechanically inoculated individually to *Nicotiana clevelandii*, a systemic host. However, plants previously inoculated with RNA-1 and then complemented with RNA-2 48 and 72 hr later became diseased within 4–5 days. In contrast, when RNA-2 was used as the primary inoculum, infections were obtained only when follow-up inoculations with RNA-1 were performed within 24 hr. RCNMV virions and genomic RNA were inoculated by electroporation into protoplasts of *N. clevelandii* and the *N. tabacum*-derived cell line BY-2 (a nonsystemic host for RCNMV). Total RNA extracts prepared from RNA-1-inoculated protoplasts after 48 and 72 hr incubation hybridized exclusively to RNA-1 specific probes, whereas RNA-2 electroporated protoplast extracts hybridized to neither RNA-1 nor RNA-2 probes. Capsid protein was synthesized in RNA-1-electroporated protoplasts but was not detected in protoplasts inoculated with RNA-2 alone. The independent replication of RCNMV RNA-1 in isolated protoplasts and results of the in planta experiments support the hypothesis that the 35-kDa polypeptide encoded by RNA-2 is involved in cell-to-cell movement. Additionally, the fact that RCNMV can replicate in the initially inoculated cells of the *N. tabacum*-derived cell line, BY-2, but does not result in systemic infection on plants of *N. tabacum* suggests that the cell-to-cell movement gene may also act as a host range determinant gene.

Additional keywords: cell-to-cell movement, electroporation, protoplasts, RNA-1 replication, suspension cell culture.

Red clover necrotic mosaic virus (RCNMV), a member of the dianthovirus group, is a 30-nm sphere with a segmented genome consisting of two single-stranded RNA species: RNA-1 (4.0 kb) and RNA-2 (1.45 kb) (22). The positive sense RNAs are both required for infectivity (7,11) and are encapsidated by 180 identical 39-kDa protein subunits encoded by RNA-1 (19,25). RNA-1 directs the synthesis of four polypeptides of molecular weights 90, 50, 39, and 27 kDa in vitro (Xiong and Lommel, *unpublished*). RNA-2 encodes a single 35-kDa polypeptide in vitro (19,25).

Except for the 39-kDa capsid protein, the function of the other gene products is not well understood. Pseudorecombination studies between RCNMV and other dianthoviruses, namely clover primary leaf necrosis virus (CPLNV), carnation ringspot virus (CRSV), and sweet clover necrotic mosaic virus (SCNMV), indicate that RNA-1 determines serological specificity (19,33). RNA-1 together with RNA-2 were shown to be involved in symptom development and lesion morphology (33,35). Evidence has also been presented that the ability of RCNMV to invade plants systemically is a property determined by RNA-2 (35). These observations suggest that the 35-kDa protein encoded by RNA-2 has a function similar to the 30- to 35-kDa proteins synthesized by a number of plant viruses believed to be involved in virus transport (1,5,17,31,46). A 30-kDa protein produced by tobacco mosaic virus (TMV) was recently demonstrated to potentiate the cell-to-cell movement of Ls1, a mutant defective in this function (6).

The putative cell-to-cell movement gene may also be involved in host range determination. It has been observed that when virus replication occurs in a resistant plant, it is limited to initially infected cells (43), suggesting that resistance may be due to the inability of the virus to invade adjacent cells. In addition, there are many examples of viruses that replicate in protoplasts of nonhost plants (36). For instance, TMV resistance in Tm-2 tomato lines is expressed at the level of the intact plant but not in protoplasts, and plant resistance is overcome only by complementation with a helper virus, which presumably provides a functional transport gene product for both viruses (45).

RCNMV-A (an Australian isolate used in these studies) systemically infects *N. clevelandii* (38) and *N. edwardsonii* (Lommel, *unpublished*) but not *N. tabacum* (38). Protoplasts from suspension cultures of the *N. tabacum*-derived cell line, BY-2 (14,48), were inoculated to determine if the nonhost phenotype is also expressed at the cellular level. BY-2 had previously been infected with TMV virions in the presence of polyethyleneamine (15) and by TMV-RNA when encapsulated in liposomes (27) or when inoculated by electroporation (32).

Electroporation is a method of gene transfer utilizing the application of brief high-voltage pulses to cells or protoplasts, resulting in transient changes in membrane permeability that allow the uptake or transport of macromolecules (3,8,12,13,37,39,49). Initially developed as a technique for the introduction of bacterial plasmid DNA into mouse cells (28,37), electroporation was recently shown to be an efficient method for inoculating virus or viral RNA into plant protoplasts derived either from mesophyll or suspension culture cells (29,30,32). Electroporation of intact plant cells with virions has also been reported (23).

Because it appears that RNA-2 is monocistronic, and that the 35-kDa protein it encodes is primarily involved in the cell-to-cell movement of RCNMV, we investigated the ability of RNA-1 to replicate independently in electroporated protoplasts. Previous studies have demonstrated that in some viruses, the larger RNA of some viruses with a divided genome can replicate in the absence of their partner RNAs (10,18,40,41).

MATERIALS AND METHODS

Virus propagation and purification. The Australian isolate of RCNMV was maintained in *N. clevelandii* A. Gray at 21 C in the greenhouse. RCNMV was purified from infected leaf tissue 7–10 days after inoculation, according to established protocols (19). Following an initial extraction in 0.2 M sodium acetate (pH 5.0), nucleoprotein was precipitated from clarified sap by the addition of one-fourth volume of 40% polyethylene glycol 6,000 in 1.0 M NaCl. Virus was concentrated and further purified by centrifugation for 2 hr at 40,000 rpm through a 30% sucrose pad. Virus concentration was determined spectrophotometrically by using E 0.1% at 260 nm of 6.4.

Extraction and separation of viral RNA. Viral RNA was isolated according to the procedure of Morris et al (24). RCNMV particles were dissociated with protease K (5 mg/ml) and 10% sodium dodecyl sulfate (SDS) followed by heating at 37 C for 30 min. An extraction medium consisting of 2× STE (0.2 M NaCl, 0.1 M Tris, pH 8.0, 2 mM EDTA), 40 mg/ml of bentonite, and 10% SDS was then added to the mixture and incubated at 60 C for 15 min. The solution was extracted with phenol, and the aqueous phase was extracted with ether several times. Viral RNA was precipitated with two volumes of ethanol in the presence of 5 M ammonium acetate.

RCNMV RNA-1 and RNA-2 were separated by fractionation in a 1.5% agarose gel buffered with 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), pH 8.0. The two RCNMV-RNA species were subsequently recovered from gel fragment slices by electroelution for 2 hr at 25–35 mA in 1× TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA), pH 7.6. Each RNA was collected in a solution of 5 M NaCl, 15% glycerol, and 0.008% bromphenol blue, extracted once in chloroform:isoamyl alcohol (24:1), and ethanol-precipitated as above.

Inoculation of RCNMV-RNA to *N. clevelandii* plants. RNA-1 and RNA-2 (2.5 μg each) were inoculated separately to *N. clevelandii* in 5 mM sodium phosphate (pH 7.0) and celite. The plants were then reinoculated with their respective partner RNAs after 24, 48, and 72 hr, kept in a growth chamber at 17–20 C, and visually assayed for systemic symptoms 1 wk later. Inoculated and noninoculated leaves of test plants were also assayed for capsid protein by enzyme-linked immunosorbent assay (ELISA) (4) and slot-blot hybridization (42).

Protoplast isolation from leaves of *N. clevelandii* and suspension culture cells of *N. tabacum* L. BY-2. Protoplasts were isolated from the youngest fully expanded leaves of 8- to 10-wk-old transplants of *N. clevelandii* that were maintained in a Conviron growth chamber at 21 C, 350 ft-c, and 16 hr daylength. Tissues were surface sterilized by dipping leaves in 15% sodium hypochlorite and 70% ethanol and then rinsed in sterile distilled water several times after each treatment. Leaves were then cut into thin strips and incubated in a petri dish containing 1.5% Cellulase "Onozuka" R-10 (Yakult Honsha Co., Japan), 0.15% Macerozyme R-10, and 0.5 M mannitol at pH 5.8. After 18–24 hr, when more than 90% of the cells had been converted to protoplasts, the suspension was filtered through Miracloth and transferred to centrifuge tubes. To obtain a clean preparation free from cellular debris, the suspension was centrifuged at low speed and the protoplast pellets washed 2–3 times with osmoticum. Occasionally, it was necessary to float the protoplasts in a babcock bottle containing 15% sucrose and 4 mM $CaCl_2$ to obtain a clean preparation.

Cells of BY-2 (14,48) were grown in MS liquid medium, pH 5.7 (26), containing 0.2 μg/ml of 2,4-D (48) at 28 C with constant agitation by using a rotary platform shaker. BY-2 was maintained by subculturing weekly with a 5% inoculum. Three days after subculture, cells were transferred to centrifuge tubes and incubated at 25 C for 6–8 hr in an enzyme solution consisting of 1.0% Cellulase R-10 and 0.1% Macerozyme R-10 dissolved in the modified MS medium supplemented with 0.4 M mannitol. For faster protoplast isolation (approximately 2 hr), 1% Cellulase RS and 0.1% Macerozyme R-10, pH 5.5, at 28 C was used.

Electroporation of protoplasts. The electroporation device consisted of a standard power supply for electrophoresis connected to a large 450-V capacitor. Discharge was regulated by a silicon-controlled rectifier. The electroporator itself was assembled by H. D. Bradshaw, Jr. (2). Electroporation in all experiments was carried out with a single 300-V pulse. Protoplasts were suspended in electroporation buffer (10 mM MES, pH 5.7, 150 mM NaCl, 4 mM $CaCl_2$, 0.4 M mannitol) at a concentration of $1–3 \times 10^6$ cells per milliliter. Electroporation was carried out with 15–20 μg/ml of RCNMV or RCNMV-RNA in 4.0-ml plastic, disposable cuvettes fitted with strips of aluminum foil on two opposite walls. After the electroporation event, cuvettes were kept on ice for 10 min, and the protoplasts were transferred to centrifuge tubes and collected. Protoplasts of *N. clevelandii* were washed twice with an incubation medium composed of 0.2 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM $CaCl_2$, 1.0 μM KI, 0.01 μM $CuSO_4$, 1 μg/ml of 2,4-D, 0.7 M mannitol, pH 7.0 (44), whereas BY-2 protoplasts were washed with the MS medium. Protoplast pellets were resuspended in their respective media to a final density of $3–5 \times 10^5$/ml and incubated for 72 hr at 17–20 C, 350 ft-c, and 16 hr daylength.

ELISA. Inoculated protoplasts were harvested at 0, 24, and 48 hr, pelleted and ground in 1× PBS (20 mM phosphate buffer, pH 7.4; 150 mM NaCl). Samples were then centrifuged and the supernatants assayed for RCNMV-specific protein by ELISA (4,20) by using anti-RCNMV IgG coupled to alkaline phosphatase.

Analysis of capsid protein by SDS-polyacrylamide gel electrophoresis (PAGE). Protoplast pellets were ground in 0.1 M Tris-HCl, pH 7.4, and centrifuged to remove cellular debris. The supernatants were concentrated to one-half their original volumes and subjected to SDS-PAGE (16) in a 5.6% stacking gel and 12.5% resolving gel. Gels were fixed in a solution of 50% methanol and 10% acetic acid and stained with 0.1% silver nitrate.

Fluorescent antibody staining. Protoplasts were decolorized and fixed with 90% ethanol before indirect fluorescent antibody staining. Dilutions used were 1:20 for anti-RCNMV serum and 1:16 for FITC-conjugated anti-rabbit immunoglobulin.

Northern hybridization analysis. Electroporated protoplasts ($1–1.5 \times 10^6$) were periodically harvested and assayed for viral RNA by slot-blot hybridization. Protoplast pellets were ground in 2× STE, 40 mg/ml of bentonite, phenol, and chloroform to extract total RNA. Following ethanol precipitation, the RNA solutions were applied directly onto GeneScreen Plus membranes (New England Nuclear Products; Du Pont Co.) by using a slot-blot apparatus. Filters were baked for 4 hr at 80 C in vacuo and hybridized at 65 C to specific probes in a solution of 0.05% SDS, 50 mM sodium phosphate pH 7.0, 2 M NaCl, and 1.5 mg/ml of salmon sperm DNA. The RNA-1 probe, a 1.2-kb cDNA clone (pRCN008) representing the 3′ terminus of RCNMV RNA-1, and the RNA-2 probe, a 1.45-kb full-length cDNA clone (pRCN005) (Xiong and Lommel, *unpublished*) were labeled with ^{32}P by nick translation (47).

RESULTS

Bioassay of RCNMV RNAs on plants of *N. clevelandii*. RNA-1 and RNA-2 were initially separated on a 1.5% agarose gel and then recovered from excised bands by electroelution. Assay of each RNA by electrophoresis showed a single band corresponding to one or the other genomic RNAs with no evidence of cross contamination (Fig. 1). In slot-blot assays, RNA-1 and RNA-2 hybridized to probes prepared to their respective RNAs only, indicating that the separated RNAs were homogeneous. Furthermore, RNA-1 or RNA-2 inoculated to seedlings of *N. clevelandii* failed to elicit systemic symptoms even after extended incubation under favorable conditions, whereas plants inoculated with unseparated RNAs became diseased within 4–5 days (Table 1). *N. clevelandii* that was previously inoculated with RNA-1 and then postinoculated with RNA-2 after 48 and 72 hr also developed systemic symptoms. On the other hand, when RNA-2 was used as the primary inoculum, positive infections were obtained only when follow-up inoculations with RNA-1 were performed within 24 hr (Table 1). No capsid protein production was detected from symptomless plants by ELISA.

Electroporation of RCNMV-RNA to *N. clevelandii* and BY-2 protoplasts. Average protoplast yields from leaves of *N. clevelandii* and BY-2 cell suspension cultures were 7×10^5 per gram of tissue and $15–20 \times 10^6$ per 50 ml of culture, respectively. Protoplast viability after isolation as determined by Evans Blue exclusion (9) was generally in the range of 89–92% for *N. clevelandii* and 95–98% for BY-2. There was a steady decline in viability in both systems as the protoplasts were maintained in culture. Viability after 72 hr incubation was around 73% for *N. clevelandii* and 85% for BY-2 protoplasts.

Indirect fluorescent antibody staining demonstrated that electroporation was an efficient method for introducing RCNMV particles and RCNMV-RNA into *N. clevelandii* and BY-2 protoplasts. The rates of infection in *N. clevelandii* were 70% by RCNMV and 81.8% by RCNMV RNA. In the case of the *N. tabacum*-derived BY-2 system, infection levels were 78.4% when the protoplasts were inoculated with RCNMV and 85.7% when inoculated with RCNMV RNA.

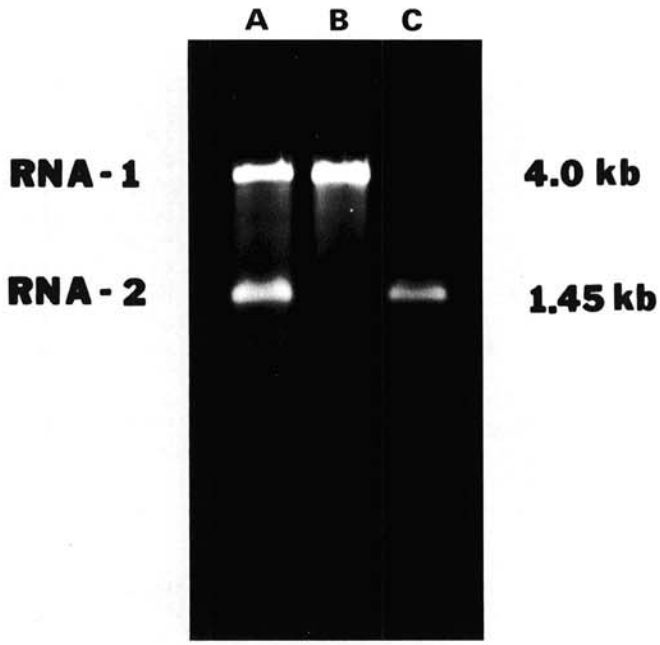

Fig. 1. Electrophoresis of RCNMV virion RNA and electrophoretically separated RNA segments in a 1% agarose gel stained with EtBr and visualized by UV transillumination. Lane A contains unfractioned RCNMV RNA liberated from purified virions. Lane B contains RCNMV RNA-1, and lane C contains RCNMV RNA-2 isolated from agarose gel slices by electroelution.

TABLE 1. Development of systemic symptoms and production of capsid protein in seedlings of *Nicotiana clevelandii* inoculated with RCNMV RNA

Inoculum	Systemic symptoms on *N. clevelandii*[a]	ELISA detection of capsid protein[b]
Buffer	−	−
RCNMV	+	+
RCNMV RNA	+	+
RCNMV RNA-1	−	−
RCNMV RNA-2	−	−
RCNMV RNA-1 then RNA-2 after:		
24 hr	−	−
48 hr	+	+
72 hr	+	+
RCNMV RNA-2 then RNA-1 after:		
24 hr	+	+
48 hr	−	−
72 hr	−	−

[a] Presence (+) or absence (−) of systemic symptoms assessed 7 days after the final inoculation.
[b] Presence (+) or absence (−) of RCNMV antigen.

Analysis of protoplast extracts by ELISA showed that RCNMV capsid protein was synthesized in both protoplast systems within the first 24 hr after inoculation with either RCNMV or RCNMV-RNA (Table 2). ELISA values increased by 40–60% after 48 hr of incubation. Capsid protein extracted from electroporated protoplasts displayed the same mobility as protein from purified virions on an SDS-polyacrylamide gel (Fig. 2).

Replication of RCNMV RNA-1 in electroporated protoplasts. *N. clevelandii* and BY-2 protoplasts electroporated with separated RCNMV-RNA were incubated for 48 hr. At various time intervals, including immediately after electroporation (0 hr), samples were withdrawn and assayed for RCNMV-RNA by slot-blot hybridization. Neither RNA-1 nor RNA-2 were detected with ^{32}P nick-translated specific probes at 0 hr (Fig. 3). However, after 24 and 48 hr of incubation, autoradiograms of total RNA from RNA-1 electroporated protoplasts showed that the extracts hybridized exclusively to the probe prepared against RNA-1 but not RNA-2.

TABLE 2. Capsid protein concentration in *Nicotiana clevelandii* and BY-2 protoplasts electroporated with RCNMV particles or combined and separated RNAs

Inoculum	Incubation (hr)[b]	ELISA absorbance values at A_{405nm}[a]	
		N. clevelandii protoplasts	BY-2 protoplasts
Buffer		0.007	0.010
RCNMV	0	0.004	0.004
	24	0.403	0.226
	48	0.718	0.565
RCNMV RNA	0	0.007	0.018
	24	0.389	0.460
	48	0.771	0.828
RCNMV RNA-1	0	0.011	0.006
	24	0.325	0.492
	48	0.563	0.627
RCNMV RNA-2	0	0.013	0.005
	24	0.008	0.002
	48	0.010	0.006

[a] ELISA values are the average of three independent experiments.
[b] 1.5×10^6 cells harvested at each time after the electroporation event followed by protein extraction.

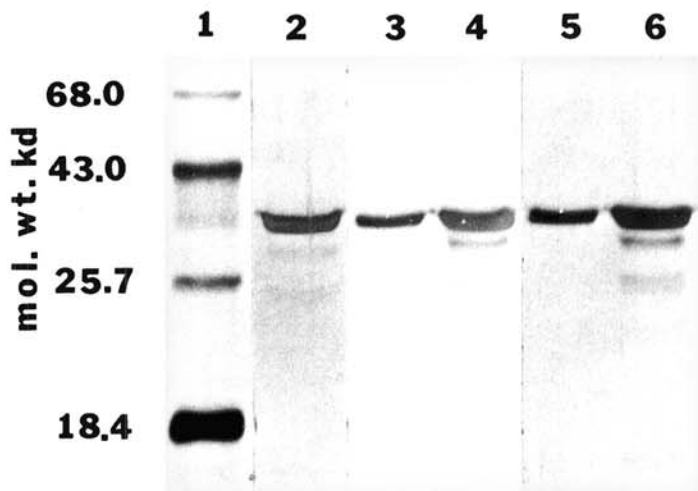

Fig. 2. Silver-stained sodium dodecyl sulfate-polyacrylamide gel (12.5%) of capsid protein extracted from RCNMV and RCNMV RNA-electroporated protoplasts. 1, Protein molecular weight standards: bovine serum albumin, 68.0 kDa; ovalbumin, 43.0 kDa; α-chymotrypsinogen, 25.7 kDa; β-lactoglobulin, 18.4 kDa. 2, Purified virus preparation of RCNMV. Capsid protein extracted from RCNMV-electroporated protoplasts of *N. clevelandii* (3), *N. tabacum* BY-2 (4). Capsid protein extracted from RCNMV RNA-electroporated protoplasts of *N. clevelandii* (5), *N. tabacum* BY-2 (6).

In contrast, extracts from RNA-2 electroporated protoplasts hybridized to neither RNA-1 nor RNA-2 specific probes.

Fluorescent antibody staining and analysis by ELISA revealed that synthesis of RCNMV capsid protein was a consequence of RNA-1 replication in the electroporated protoplasts (Table 2). The efficiency of infection by RNA-1 approached that of RCNMV-RNA. Likewise, ELISA absorbance values for RNA-1 inoculated protoplasts were comparable to those of protoplasts electroporated with unseparated RCNMV-RNA.

DISCUSSION

RCNMV RNA-1 inoculated into plants of *N. clevelandii* did not produce visible disease symptoms and was not recovered from mechanically inoculated leaves. Systemic symptoms were expressed only upon complementation of RNA-1 with RNA-2. These results indicate that RNA-1 persisted in the inoculated cells but its replication was undetected, due perhaps to the low efficiency of infection associated with mechanical inoculation (21). It could also be due to the inability of RNA-1 to invade adjacent cells and move systemically in the absence of RNA-2, suggesting that RNA-2 contributes the virus transport function. These observations are consistent with findings based on pseudo-recombination experiments that RNA-2 is involved in the cell-to-cell movement of RCNMV (35). RNA-2 encodes a 35-kDa polypeptide (19,25) that may be functionally analogous to the TMV 30-kDa movement protein (6).

Electroporation proved to be an efficient method for introducing both RCNMV particles and RNA into protoplasts. The procedure was simple, rapid, and gave reproducible results. The infection rates obtained in this study are comparable to those observed by Okada et al (32) for BY-2 protoplasts electroporated with either TMV-RNA or CMV-RNA where up to 80% of the cells became infected. Nishiguchi et al (30) reported an 80–90% infection of tobacco (*N. tabacum* L. 'Petit Havana SR1') mesophyll protoplasts by using TMV particles as inoculum. Analyses of electroporated protoplast extracts by northern hybridization confirmed the ability of RNA-1 to replicate autonomously in both the systems of *N. clevelandii* and *N. tabacum* BY-2. In addition, fluorescent antibody staining and ELISA revealed the production of capsid protein in the RNA-1 inoculated protoplasts. It has been previously demonstrated through peptide mapping and immunoprecipitation that RNA-1 encodes the 39-kDa RCNMV capsid protein (25). Evidence has also been presented that serological specificity is conferred by RNA-1 (19,33,35). These results indicate that the entire replicative function and capsid protein production of RCNMV is associated exclusively with RNA-1 and that no transacting RNA-2 factors are involved. We have shown by electrophoretic and northern hybridization analyses that there was no cross contamination between the RNA species used as inocula. That no symptoms were observed on plants inoculated with the separated RNAs even after prolonged incubation under favorable conditions is further proof of the biological purity of the RNA samples. Because RNA-1 replicates independently in isolated protoplasts but is capable of systemic infection only in the presence of RNA-2, it appears that the singular role of RNA-2 is to facilitate the cell-to-cell movement of RCNMV.

The replication of RNA-1 in BY-2, a cell line derived from *N. tabacum*, a nonhost (38), suggests that, in addition to the transport function, RNA-2 may also act as a host range determinant. Taliansky et al (45) reported that in Tm-2 tomato lines, resistance to TMV at the plant level could be overcome by preinfection with a helper virus with a functional transport gene, an indication that the "movement" gene also controls virus host range for TMV. In contrast, Tm-1 lines that exhibit TMV resistance both at the plant and protoplast levels remain unaffected by preinfection.

While this manuscript was in preparation, Osman and Buck (34) reported that RCNMV RNA-1 inoculated in the presence of polyethylene glycol replicated independently and synthesized virus capsid protein and particles in cowpea mesophyll protoplasts. Our studies with electroporated protoplasts of *N. clevelandii* and *N. tabacum* BY-2 confirm their results. Autonomous replication of the larger RNA is not unique to RCNMV and has been demonstrated in some members of the tobraviruses (18,41), comoviruses (10), and nepoviruses (40). However, in these viruses the coat protein gene resides in the separate partner RNA, and hence during RNA replication no coat protein synthesis occurs.

Results of our in planta and protoplast experiments support the hypothesis that the 35-kDa polypeptide encoded by RNA-2 is involved in the cell-to-cell movement of RCNMV and possibly in host range determination. RCNMV may be an ideal system for studying proteins involved in plant virus transport because it is encoded on a separate RNA that appears to be monocistronic. It can therefore be manipulated independently of all other viral genes.

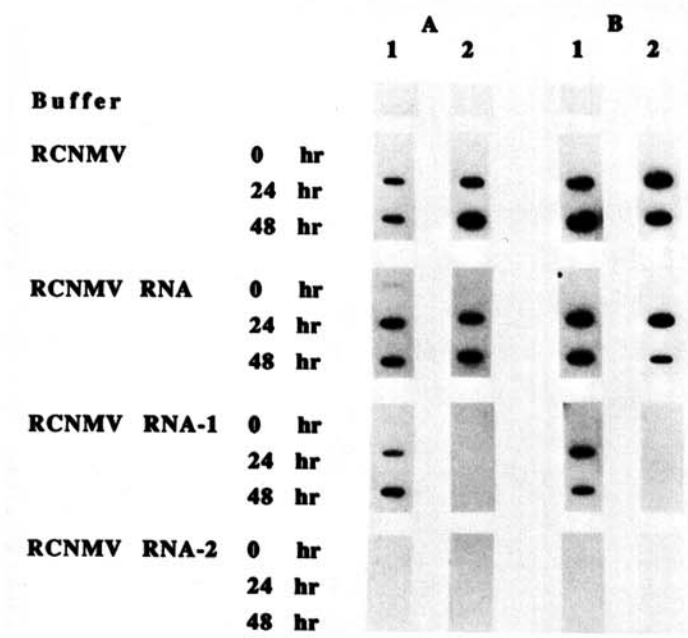

Fig. 3. Northen hybridization analysis of protoplasts electroporated with fractionated RCNMV RNA. Total RNA was extracted from electroporated *N. clevelandii* (**A**) and *N. tabacum* BY-2 (**B**) protoplasts ($1–1.5 \times 10^6$) at 0, 24, and 48 hr after inoculation and applied to GeneScreen Plus membranes by using a slot-blot apparatus. The filters were hybridized to ^{32}P-labeled probes prepared by nick translation. The RNA-1 probe (1) is a 1.2-kb cDNA clone (pRCN008) representing the 3′ end of RNA-1, and the RNA-2 probe (2) is a 1.45-kb full-length cDNA clone (pRCN005).

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