CHAPTER 10

Dianthoviruses: Properties, Molecular Biology, Ecology, and Control

R. I. HAMILTON AND J. H. TREMAINE

I. INTRODUCTION

The genus *Dianthovirus* consists of three definitive species: carnation ringspot virus (CRSV) (the type species), red clover necrotic mosaic virus (RCNMV), and sweet clover necrotic mosaic virus (SCNMV) (Francki *et al.*, 1991). A possible fourth species is furcraea necrotic streak virus (FNSV), which is serologically related to RCNMV and hybridizes with cDNA clones to each of the two RCNMV genomic RNA species (Morales *et al.*, 1992). The genus name is derived from *Dianthus*, the generic name of carnation (*D. caryophyllus*), which is the most common natural host of CRSV. Dianthovirus particles are isometric, 33 nm in diameter, and sediment as a single species with a sedimentation coefficient ($s_{20,w}$) of about 133 *S* at pH 5.0. They contain a single capsid protein with molecular weight of approximately 37×10^3 and two major genomic RNA species with molecular weights of approximately 1.5×10^6 (RNA-1) and 0.5×10^6 (RNA-2). Dianthovirus particles are stable and easily purified with yields up to 100 mg/kg of infected tissue, and are thus well suited to studies of virus structure and replication. However, di-

R. I. HAMILTON AND J. H. TREMAINE • Pacific Agriculture Research Centre, Agriculture and Agri-Food Canada, Vancouver, British Columbia, Canada V6T 1X2.

B. D. Harrison et al. (eds.), *The Plant Viruses*
© Springer Science+Business Media New York 1996

anthoviruses have not been studied as intensively as other plant viruses with bipartite genomes, probably because they do not usually cause serious crop losses. Two comprehensive reviews on the properties of the dianthoviruses have been published within the last few years (Hiruki, 1987; Giesman-Cookmeyer *et al.*, 1995) that attest to recent interest in this genus of plant viruses.

Noordam *et al.* (1951) were probably the first to isolate what is now known as CRSV from carnation showing mosaic symptoms, and named it carnation mosaic virus. Kassanis (1955) studied a carnation virus that was similar if not identical to that of Noordam *et al.* (1951) but preferred the name CRSV to describe the symptoms induced in seedling carnation and to avoid confusion with a different virus named carnation mosaic virus by American workers. Using serology, host range, thermal inactivation point, longevity *in vitro*, and electron microscopy, Kassanis (1955) distinguished CRSV from carnation mottle virus, another isometric virus commonly found in carnation. Hollings and Stone (1970) found that CRSV did not react with antisera to any of 35 other isometric viruses.

The first description of symptoms in red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) infected by RCNMV was by Musil and Matisova (1967) in Czechoslovakia. Musil (1969a,b) isolated RCNMV and distinguished it from other clover viruses by host range, physical properties, electron microscopy, and serology. SCNMV (Hiruki *et al.*, 1984b; Hiruki, 1986b) was first isolated from sweet clover (*Melilotus officinalis*) exhibiting systemic mosaic, ringspots, and veinal necrosis near Grand Prairie and Athabasca in Alberta, Canada (Hiruki, 1986a). FNSV, the causal virus of "macana" or necrotic streak of fique (*Furcraea macrophylla* and *F. cabuya*), a major fiber crop in Colombia, South America, was first reported in the Department of Antioquia in western Colombia, but it is now distributed throughout the main fique-production areas of the Andean regions of Colombia (Dabek and Castaño, 1978).

The first report pointing to a bipartite genome for CRSV was the observation of two distinct RNA components in density gradient centrifugation of the products of sodium dodecyl sulfate (SDS) dissociation of virus particles (Tremaine and Ronald, 1976). Ragetli and Elder (1977) reported a similar result on SDS treatment of clover primary leaf necrosis virus, first described as a new virus but now considered a strain of RCNMV (Rao and Hiruki, 1985) and designated here as RCNMV-Can. Dodds *et al.* (1977) established that both RNA species of CRSV were required for infectivity, thus proving the bipartite nature of the dianthovirus genome.

II. HOST RANGE, SYMPTOMS, AND GEOGRAPHICAL DISTRIBUTION

CRSV has caused serious problems in carnations grown by vegetative propagation (Kassanis, 1955; Hollings and Stone, 1970; Lommel *et al.*, 1983).

Symptoms in naturally infected carnation and sweet william (*D. barbatus*) include curling or distortion of leaves, occasionally with tip necrosis (Hollings and Stone, 1965). Flowers are often distorted, of poor quality, and are thus unmarketable. The experimental host range of CRSV includes over 130 species in 25 dicotyledonous families (Tremaine and Dodds, 1985). With the establishment of virus-free certified plant propagation material and the use of serological indexing and strict sanitation procedures, CRSV has been virtually eliminated from commercial carnation plantings in western Europe (Sparnaaij, 1983), North America (Lommel *et al.*, 1983), and Israel (Mor, 1983), but a high proportion (33–53%) of Sim, Mediterranean, and spray types of carnation were infected in commercial production nurseries near Bogota, Colombia (Valenzuela and Pizano, 1992).

CRSV has been mechanically transmitted to herbaceous hosts from pear (*Pyrus communis*) trees with stony pit disease, from apple (*Malus sylvestris*) and sour cherry (*Prunus cerasus*) trees with decline syndromes (Richter *et al.*, 1978; Kleinhempel *et al.*, 1980), and from plum (*Prunus domestica*) trees (Casper, 1976). It has also been detected serologically in petals, leaves, fruits, roots, and cambium tissues from apple trees with apple spy decline and pear trees with pear stony pit and vein yellows diseases. CRSV was also mechanically transmitted to some apple virus indicator plants as evidenced by reisolation on herbaceous hosts. However, the significance of CRSV in the etiology of fruit tree diseases remains uncertain.

Weeds, too, were naturally infected with CRSV in these CRSV-infected orchards. *Stellaria media* was the species most frequently infected, and in tests with 1000 plants, CRSV infection occurred in 8% at some individual sites, and in 32 and 70% at two sites in a 5-year-old nursery containing CRSV-infected apple trees. CRSV infection was detected in 21 of 40 *Urtica urens* plants and in 4 of 40 *Poa annua* plants in another CRSV-infected apple orchard (Kleinhempel *et al.*, 1980). CRSV-infected plants of all three species were symptomless. CRSV infection of *S. media* in vineyards has also been reported (Rüdel *et al.*, 1977). The role of weeds as sources of virus for infection of tree fruits and grapes remains to be determined.

RCNMV infects red clover, alfalfa, white clover (*Trifolium repens*), and sweet clover growing as field crops or in pastures, and has been reported in northern Europe, Australia, New Zealand, and Canada (Hollings and Stone, 1977). In naturally infected red clover, the leaves show a mild to severe mosaic, with mild to severe necrosis of the veins accompanied by leaf distortion, and the plants show moderate to severe stunting in the winter (Musil, 1969a,b). In the summer these symptoms are wholly or partially masked, particularly in newly infected plants. Inoculated red clover plants show similar symptoms but local necrotic lesions are prominent. Sweet clover plants show mild to severe systemic mosaic symptoms, probably dependent on the season in which the plants were infected or observed. Okuno *et al.* (1983), using controlled growth chambers maintained at 17 °C and 26 °C in a study of pseudorecombinants of three strains of RCNMV, determined that temperature was critical in obtaining reproducible symptoms for use as

genomic markers. The wide variation in symptomatology and host range reported for RCNMV by Musil (1969a), Gerhardson and Lindsten (1973), Hollings and Stone (1977), Ragetli and Elder (1977), and Hiruki (1987) was probably due in part to studies being done with plants at different temperatures or with less strictly regulated temperatures. However, virus strain differences (Hiruki, 1987), differences in susceptibility of plant cultivars to RCNMV (Bowen and Plumb, 1979), and differences in inoculation technique were probably involved as well.

The natural host of SCNMV is sweet clover. The virus was not detected in alsike clover (*Trifolium hybridum*), red clover, or crown vetch (*Coronilla varia*) growing on sites containing SCNMV-infected sweet clover (Hiruki, 1986a), but a serologically distinct strain was subsequently isolated from alfalfa showing very mild chlorosis and mild stunting in the same area (Inouye and Hiruki, 1985; Pappu *et al.*, 1988). The geographical distribution of both strains appears to be limited to the province of Alberta, Canada.

Fique plants infected for a prolonged time with FNSV are stunted and the leaves show chlorotic streaks on both sides, which later coalesce and become necrotic. Chronically infected plants usually die (Dabek and Castaño, 1978). The natural host range is restricted to fique, but the virus can be mechanically inoculated to *F. selloa* var. *marginata* and sisal (*Agave sisalana*).

Although natural infections are limited to a few species, the experimental host ranges of CRSV, RCNMV, and SCNMV include many plant species in at least 25 dicotyledonous families (Hollings and Stone, 1970, 1977; Hiruki *et al.*, 1984b; Pappu *et al.*, 1988). RCNMV does not infect carnation or sweet william, but CRSV infects crimson clover (*Trifolium incarnatum*) systemically (H. W. J. Ragetli, unpublished results). The alfalfa isolate of SCNMV could be distinguished from the sweet clover isolate by its inability to infect tomato (*Lycopersicon esculentum*) (Pappu *et al.*, 1988). Plants naturally infected by dianthoviruses typically exhibit mild mosaic symptoms that are often alleviated at temperatures above 20 °C.

III. VIRION PROPERTIES

A. Structure

Dianthovirus particles are probably composed of either a single RNA-1 molecule (molecular weight 1.5×10^6) or three RNA-2 molecules (molecular weight 0.5×10^6), encapsidated by 180 protein subunit molecules of 37 to 39 kDa arranged in a $T = 3$ structure (Fig. 1) The weight of such particles would thus be 8.2 to 8.5 MDa. The particle weight of CRSV determined by sedimentation–diffusion is 7.1 MDa (Kalmakoff and Tremaine, 1967). The larger values seem more consistent with those for the tombusviruses, which are similar in size but have a monopartite genome.

Most freshly prepared dianthovirus particle preparations contain a sin-

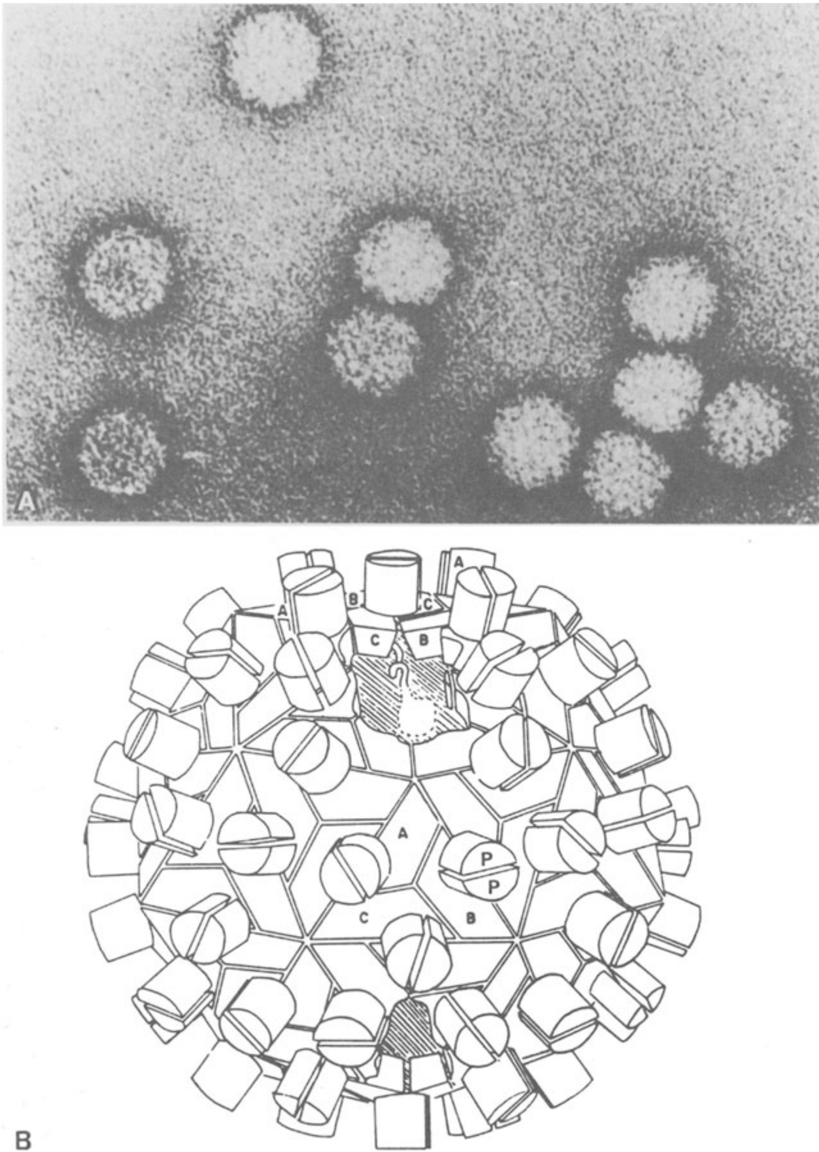

FIGURE 1. Structure of dianthovirus virions. (A) Electron micrograph of purified RCNMV particles in negative stain. The particles are approximately 33 nm in diameter. (B) Model of dianthovirus virus particle based on crystallographic analysis of turnip crinkle and tomato bushy stunt tombusviruses. Individual capsid subunits are labeled A, B, and C, depending on their packing environment. Note the granular nature of the surface of the virus particles shown in (A), which is presumably due to the protruding domains (P) of the capsid subunits. (After Giesman-Cookmeyer *et al.*, 1995.)

gle 37- to 39-kDa protein component (Barton, 1981; Lommel, 1983; Hiruki *et al.*, 1984b; Tremaine and Dodds, 1985). However, some preparations stored at 4 °C contain 36-kDa and 34-kDa proteins, presumably formed by proteolytic degradation of the 37- to 39-kDa protein. This limited proteolysis occurs to a greater extent with CRSV than with RCNMV (Barton, 1981; Tremaine and Dodds, 1985; J. H. Tremaine, unpublished results). The amino acid compositions of four strains of CRSV reported by Kalmakoff and Tremaine (1967) and Tremaine *et al.* (1984) (Table I) were of preparations containing a single 38-kDa protein. Results of similar analyses (J. H. Tremaine, unpublished results) of RCNMV-Can (Ragetli and Elder, 1977) and RCNMV-V from Victoria, Australia (Gould *et al.*, 1981), here designated as RCNMV-Aus, are also shown in Table I. There are no major differences in amino acid composition between the two RCNMV strains or among the four CRSV strains. The amino acid compositions of the individual dianthoviruses have many differences but are basically similar.

TABLE I. Number of Amino Acid Residues in Capsid Proteins of Two Strains (Aus and Can) of Red Clover Necrotic Mosaic Virus (RCNMV) and Four Strains (R, N, A, K) of Carnation Ringspot Virus (CRSV)

	RCNMV		CRSV			
Amino Acid	Aus[a]	Can[a]	R[b]	N[b]	A[b]	K[c]
Lys	16	14	15	16	15	14
His	2	2	2	2	3	2
Arg	18	21	15	17	16	16
Asx	32	31	33	35	34	34
Thr	34	32	38	38	38	37
Ser	38	37	39	38	38	37
Glu	28	26	22	23	23	23
Pro	18	23	19	19	17	20
Gly	25	28	23	24	23	20
Ala	30	24	24	26	27	24
Cys	5	6	2	3	3	3
Val	34	30	36	30	32	36
Met	3	3	6	4	6	7
Ile	14	16	16	15	14	16
Leu	24	26	27	27	27	26
Tyr	11	11	15	15	15	16
Phe	12	14	13	12	12	12
Trp	nr[d]	nr	3	3	4	4
Total	344	344	348	347	347	347

[a]J. H. Tremaine (unpublished data).
[b]Tremaine *et al.* (1984).
[c]Kalmakoff and Tremaine (1967).
[d]nr, Not reported.

The molecular weights of dianthovirus RNA species, estimated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions, are 1.5×10^6 (RNA-1) and 0.5×10^6 (RNA-2) for both RCNMV (Gould *et al.*, 1981) and CRSV (Lommel, 1983). By cDNA hybridization studies, Gould *et al.* (1981) established that RCNMV RNA-1 and RNA-2 are unique RNA species with little or no sequence homology. Lommel (1983) did not detect sequence homology between the RNA species of CRSV and RCNMV, but significant sequence homology has been reported at the amino acid level for several gene products of CRSV, RCNMV, and SCNMV (Ge *et al.*, 1992; Kendall and Lommel, 1992; Ryabov *et al.*, 1994). The significant sequence homology between RCNMV and FNSV (Morales *et al.*, 1992) suggests that FNSV may be a strain of RCNMV.

The nucleotide composition of unfractionated CRSV RNA is G, 26%; A, 27%; C, 23%; and U, 24% (Kalmakoff and Tremaine, 1967). The lack of retention of CRSV or RCNMV RNA molecules on oligo(dT) cellulose columns indicates the absence of 3′ poly(A) sequences (Lommel, 1983; Tremaine and Dodds, 1985), and no poly(A) tracts have been detected in dianthovirus genomes by nucleotide sequence analysis. The stem-loop regions at the 3′ end of the dianthovirus RNA species, which are involved in the interaction with the virus-specific RNA polymerase, must be similar, if not identical, because pseudorecombinants made from RNA-1 of RCNMV and RNA-2 of CRSV (Lommel, 1983), and both possible pseudorecombinants of RCNMV and SCNMV (Okuno *et al.*, 1983), are infective, usually inducing local or systemic symptoms similar to those induced by RNA-1 of the parental virus.

CRSV preparations with native 38-kDa protein give a single band with a buoyant density of 1.366 g cm^{-3} in CsCl gradient centrifugation. Additional components with buoyant densities of 1.369 and 1.374 g cm^{-3} are present in virus preparations containing partially proteolyzed protein (Tremaine and Dodds, 1985). The major component of RCNMV has a buoyant density of 1.37 g cm^{-3} in CsCl, but a minor component with a greater density has also been observed (Gould *et al.*, 1981). This indicates that some viruslike particles contain a considerable amount of the lower-molecular-weight protein. Infectivity is associated with the single buoyant density component, which indicates that the particles containing one RNA-1 molecule have the same density as those containing three RNA-2 molecules, and therefore the size of RNA-2 must be one third that of RNA-1. Subgenomic RNA species that code for the coat protein may also be encapsidated in RCNMV particles (Morris-Krsinich *et al.*, 1983).

Direct evidence of the packaging of one molecule of RNA-1 in one particle type and three molecules of RNA-2 in another particle type is not obtainable because of the single buoyant density. Packaging of both RNA species in the same particle as occurs with Nodamura virus, a bipartite genome virus of invertebrates (Longworth, 1978), is improbable because the molar ratios of RNA-1:RNA-2 in CRSV are 1:1.7 to 1:2.8. Moreover, most of

the RNA released from CRSV particles in 0.01% SDS is RNA-2, and RNA-1 is released at higher SDS concentrations (Tremaine and Ronald, 1976).

The isometric particles of CRSV, FNSV, RCNMV, and SCNMV are 34–35 nm in diameter (Tremaine and Ronald, 1976; Hatta and Francki, 1984; Hiruki *et al.*, 1984b; Morales *et al.*, 1992). In a comparative electron microscopy study of isometric particles from ten virus groups, Hatta and Francki (1984) distinguished RCNMV from all viruses except tomato bushy stunt tombusvirus (TBSV) on the basis of size (34.2 nm), round particle outline, and rough surface appearance. CRSV particles appear similar to those of RCNMV but the particle surface is less rough. Orlova *et al.* (1980) noted that the capsomeric structure of CRSV is very similar to that of TBSV, which consists of 90 dimers and is characterized by 5:3:2 symmetry. In two-dimensional crystal specimens of CRSV the distance between particle centers in the hexagonal packing of particles is 34 to 35 nm.

B. Infectivity–Dilution Curves

Infectivity dilution data for CRSV (J. H. Tremaine and W. P. Ronald, unpublished results) provide another line of evidence for the independent packaging of RNA-1 and RNA-2. These data were fitted to four equations: Eq. (1), a single-hit curve for one particle; Eq. (2), a two-hit curve for identical particles; Eq. (3), a two-hit curve for two distinct particle types A and B in equal quantities; and Eq. (4), a two-hit curve for two distinct particle types A and B in any ratio. These equations are:

$$y = N(1 - e^{cx}) \tag{1}$$

$$y = N[(1 - e^{cx}) - cx(e^{cx})] \tag{2}$$

$$y = N(1 - e^{cx})^2 \tag{3}$$

$$y = N(1 - e^{ax})(1 - e^{bx}) \tag{4}$$

where y = number of lesions, N = maximum possible number of lesions, c = total number of virus particles, x = dilution, and a and b are the numbers of the A and B particle types, respectively. After fitting the experimental data to the four equations, the summation of squared residuals was 21.5 for Eq. (1), 8.7 for Eq. (2), 6.0 for Eq. (3), and 1.9 for Eq. (4). The ratio of a/b derived from the best fit to Eq. (4) was 4:1. The molar quantity of RNA-2 found on SDS dissociation of this virus preparation was 66% of that of RNA-1. If three RNA-2 molecules were packaged in a single particle, the ratio of particles containing RNA-1 to particles containing RNA-2 would be 4.5 to 1. Therefore, the infectivity–dilution data are best approximated by Eq. (4), i.e., two distinct particle types are required for infection.

C. Virion Stability

Dianthovirus particles have many characteristics in common with bromovirus particles, which are stabilized by RNA–protein interactions and pH-dependent protein–protein interactions (Kaper, 1975). However, some strains of CRSV are more stable than others, and RCNMV is more stable than CRSV. This variation of stability within members of the same virus group is not unexpected. For example, nitrous acid mutants of cowpea chlorotic mottle bromovirus have been isolated that are either much more stable or much less stable than the type virus (Lane, 1974).

CRSV particles swell slowly on change of pH from 5.0 to 7.5 (Tremaine and Ronald, 1976, and unpublished results; Kühne and Eisbein, 1983). Three naturally occurring CRSV strains, designated N, A, and R (Tremaine *et al.*, 1976), differ in the proportion of virus particles swollen 1 hr after adjustment of the pH to 7.0 or 7.5. Most particles of all strains are swollen after 24 hr at pH 7.5. EDTA increases the rate of swelling, but Mg or Ca ions prevent it. When swollen CRSV-N particles are treated with ribonuclease, isometric particles of 22-nm diameter (probably $T = 1$ structures), ellipsoidal particles 22×33 nm and double-shelled particles of 33 nm diameter are formed (J. H. Tremaine and W. P. Ronald, unpublished results). Addition of trypsin to CRSV-N at pH 5.0 has no effect, but the 38-kDa protein is cleaved to 36-kDa and 34-kDa proteins at pH 7.0 to 8.0 and into 20-kDa to 26-kDa species at pH 9.0 (J. H. Tremaine and W. P. Ronald, unpublished results). The presence of contaminating plant proteases in some virus preparations is indicated by a limited proteolysis on storage (i.e., presence of 36-kDa and 34-kDa proteins), and this proteolysis is more evident in preparations stored at pH 7.0 than at pH 5.0 (Barton, 1981; J. H. Tremaine and W. P. Ronald, unpublished results). The purification of CRSV by ion exchange chromatography (Tremaine, 1961) or by CsCl density gradient centrifugation reduces proteolysis on subsequent storage.

Approximately 30% of the particles in a preparation of RCNMV-Can were swollen after 1 hr at pH 7.0 (J. H. Tremaine and W. P. Ronald, unpublished results). However, Lommel (1983) did not detect swelling of RCNMV-Aus at pH 7.0. Barton (1981) did not detect limited proteolysis of RCNMV in stored preparations at pH 7.0, but we have noted proteolysis on storage, as have Gould *et al.* (1981).

After 24 hr, approximately 50% of CRSV-N particles dissociate into RNA and protein components in 0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, and 1 M NaCl (J. H. Tremaine and W. P. Ronald, unpublished results). The virus particles can be reconstituted from RNA and protein components by dialysis against 0.1 M Tris-acetate buffer, pH 5.0, containing 1 mM $CaCl_2$. The particles are heterogeneous and some geminate particles are formed. The protein did not assemble into empty capsids at pH values from 4 to 8. However, in the presence of a nucleating agent, sodium dextran sulfate (NDS), the protein formed misshapen particles of 20 to 33 nm diameter.

CRSV strains differ in the readiness with which their particles dissociate into RNA and protein after treatment at room temperature for 30 min with SDS at pH 7.0 (Tremaine and Ronald, 1976). Particles of strain N are almost completely dissociated by 0.4% SDS at pH 5.0 and by 0.1% SDS at pH 7, but those of strains R and A were almost completely unaffected at pH 5 by SDS concentrations up to 15%. However, in 0.05% SDS at pH 7, some particles dissociated, some were unaffected, and others swelled to 49 nm diameter but remained resistant to higher SDS concentrations. RCNMV-Can particles, too, are resistant to dissociation by SDS at pH 5.0. Approximately 20% of the virus particles were swollen after 1 hr at pH 7.5 and 50% of the virus particles were dissociated in 1% SDS (J. H. Tremaine and W. P. Ronald, unpublished results). Results from pseudorecombination experiments between CRSV-A and CRSV-N indicate that the capsid protein gene, which is located on RNA-1, probably controls sensitivity of CRSV to SDS (Dodds *et al.*, 1977).

Virions stabilized by RNA–protein interactions are very much like polyelectrolyte complexes when in the swollen condition. When increasing amounts of NDS were added to CRSV at pH 7.5, increasing amounts of RNA were liberated until at an NDS–virus ratio of 1:4 by weight, nearly all the virus particles were dissociated (Tremaine *et al.*, 1983). The acidic polyelectrolyte NDS has a greater affinity for the coat protein than does the RNA. Isometric viruslike particles (22 nm or 33 nm diameter) with characteristics of $T = 1$ and $T = 3$ particles, respectively, formed of NDS and the CRSV protein were found in these preparations by electron microscopy. NDS had no effect on unswollen virus particles at pH 5.

The addition of one part NDS to four parts RCNMV-Can by weight at pH 7.5 dissociated only 20% of the virus particles; 60% of the particles remained stable; and 20% formed particles that were greatly swollen (J. H. Tremaine and W. P. Ronald, unpublished results). All virus particles were dissociated at pH 8.25, but capsid protein subunits reassembled with NDS to form both $T = 1$ and $T = 3$ particles, which can be readily distinguished from virus particles by electron microscopy.

D. Aggregation

Particles of CRSV strains N, A, and R differ in their tendency to aggregate in suspension (Tremaine *et al.*, 1976). Strain A forms clusters of 12 virus particles and aggregates of linked clusters following high-speed centrifugation. This type of aggregation has been observed with a strain of tobacco necrosis satellite virus and with radish mosaic C comovirus (Kassanis and Woods, 1968; Kassanis *et al.*, 1973). Virus particles of strains R and N of CRSV aggregated at 25 °C and 40 °C, respectively, but disaggregated to monomers at lower temperatures, and the effects of virus concentration and some chemicals on this endothermic process were studied by Kühne *et al.* (1983), Tremaine *et al.* (1984), and Tremaine and Ronald (1985).

E. Serological Properties

Tremaine *et al.* (1976) readily detected serological differences between A and either N or R strains of CRSV in immunodiffusion tests. A serological reaction of partial identity between strains N and R was consistently observed by immunodiffusion against CRSV-N antiserum at low dilution (1:2), and this result was confirmed by intragel adsorption tests (Tremaine *et al.*, 1984). A CRSV isolate from pear appeared identical to a carnation isolate (Kleinhempel *et al.*, 1980); however, extensive tests for serological differences were not done.

Musil and Gallo (1982) distinguished three serotypes of RCNMV: A (RCNMV-TpM34) and B (RCNMV-TpM48), both from Czechoslovakia (Musil, 1969b), and C (RCNMV-Sw) from Sweden (Gerhardson and Lindsten, 1973). Musil *et al.* (1982) examined 34 RCNMV isolates from different regions of Czechoslovakia and found A, B and C serotypes separately and in mixtures. Their distribution was not limited to definite geographical areas. These serotypes had different electrophoretic mobilities and could be distinguished by immunoelectrophoresis with mixed antisera to serotypes A, B, and C.

RCNMV isolates G from England, S from Scotland, and Aus from Australia have strong cross-reactions with antiserum to serotype B (Hollings and Stone, 1977; Gould *et al.*, 1981). However, a serological analysis by the intragel adsorption test of six RCNMV isolates, Aus, Can, Eng (Bowen and Plumb, 1979), Sw (serotype C), TpM34 (serotype A), and TpM48 (serotype B) clearly demonstrated that RCNMV-Can belongs to serotype B and that RCNMV-Aus and RCNMV-Eng are members of a new serotype, D (Rao *et al.*, (1987). Interestingly, adsorption of the RCNMV-Aus antiserum with homologous antigen or the heterologous RCNMV-Eng antigen completely removed the homologous antibodies, but the remaining antibodies were heterospecific, reacting with RCNMV-Can, -Sw, and -TpM48 but not with the homologous antigen. None of the antisera to the other viruses contained heterospecific antibodies that occur in some polyclonal antisera to tobacco mosaic tobamovirus (TMV) (Van Regenmortel, 1982).

Hiruki *et al.* (1984a) found that direct, double antibody sandwich enzyme-linked immunosorbent assay (ELISA) failed to detect any cross-reaction between antigens of RCNMV, SCNMV, or CRSV and their antisera. In indirect ELISA, however, where the combination of bound antigen and antivirus antibody was detected by antiglobulin antibodies from a different animal species, an antiserum to RCNMV-Can gave weak cross-reactions with two other RCNMV strains and with CRSV and SCNMV, and an antiserum to CRSV gave somewhat weaker cross-reactions with all three strains of RCNMV and with SCNMV. Different results in direct and indirect ELISA are not unexpected because the conformation of plate-bound antigens is often more distorted than that of antibody-bound antigens, resulting in exposure of different epitopes in the protein for subsequent binding of antibodies and

giving a consequential increase in cross-reactivity (Van Regenmortel and Burckard, 1980). Cross-reactions between CRSV and RCNMV were much greater with RCNMV and CRSV antisera, respectively, in indirect ELISA when the dissociated capsid proteins were used as antigens (Tremaine and Dodds, 1985). Hiruki and Figueiredo (1985) found that two SCNMV monoclonal antibodies consistently cross-reacted with CRSV, RCNMV-Aus, and RCNMV-Eng, but cross-reacted poorly with RCNMV-Can and RCNMV-TpM48 from Czechoslovakia.

Bercks and Querfurth (1972) detected weak cross-reactions between particles of some tymoviruses and one CRSV antiserum in gel diffusion tests in 0.25 M phosphate buffer. This may be attributable to formation of nonspecific precipitates in this buffer in the absence of NaCl (Altschuh and Van Regenmortel, 1983). Given the many differences in the properties of dianthoviruses and tymoviruses, it is highly unlikely that their capsids are serologically related.

F. Electrophoretic Mobility

The electrophoretic mobility of CRSV particles was determined in free boundary Tiselius electrophoresis in the pH range 4 to 8 (J. Kalmakoff and J. H. Tremaine, unpublished results). The virus moved as a single discrete boundary in the pH range 4 to 6.5 with an isoelectric point at pH 5.1. As the pH was increased to 7.0 and 7.5, the moving boundary spread to the anode and continued to spread when the polarity was reversed. Migration in agarose was similar. Kühne *et al.* (1983) also obtained discrete, nonspreading migration of CRSV in immunoelectrophoresis at pH 5.0 and 6.5 in 0.01 M phosphate buffers containing 0.001 M $MgSO_4$. Electrophoretic spreading may be a result of virion swelling, as indicated by electrophoresis studies with Ca or Mg ions at pH 7 to 8, or to changes in the capsid induced by limited proteolysis of the virus.

Gallo and Musil (1984a) determined the electrophoretic migration of serotypes A, B, and C of RCNMV in agarose containing phosphate at pH 7.2 and 8.0, or Tris-HCl, sodium diethylbarbiturate, or HEPES at pH 7.2 and 8.6, with buffer molarities of 0.1. 0.01 and 0.001 M. Migration at all molarities in HEPES was cathodic. Migration in most other buffers was anodic at 0.1 M, more slowly anodic at 0.01 M, but cathodic at 0.001 M. In isoelectric focusing in agarose containing 6.3% Pharmalyte 3-10, the isoelectric points of the A, B, and C serotypes were pH 5.0, 4.8, and 4.6 respectively, but the A and B serotypes each contained a minor component with an isoelectric point of pH 4.2 (Gallo and Musil, 1984b). The minor components may be virions in which the coat protein had been partially proteolyzed. These isoelectric points indicate that the virion should migrate anodically above pH 5 in agarose electrophoresis. The effect of buffer molarity on this migration may have been induced by swelling of the virion. On electrophoresis in Ionagar #2 in

0.03 M phosphate buffer, pH 7.6, Hollings and Stone (1977) noted that five RCNMV isolates migrated to the cathode: C and A migrated slowly, B more rapidly, and G and S much more rapidly. Assuming considerable electroendosmotic flow in agar with a high sulfate content, the migration of A, B, and C is consistent with the results of Gallo and Musil (1984a) in phosphate buffers. The movement of G and S indicates they are similar to B but with a lower negative charge.

A comparative study of the electrophoretic mobility of seven isolates of the definitive dianthoviruses in agarose under nondenaturing conditions in 0.01 M phosphate, pH 7.0, showed considerable and reproducible variation between isolates of the same virus (Pappu and Hiruki, 1989). All viruses moved anodically: CRSV-N moved slowest; RCNMV-TpM34 and RCNMV-TpM48 moved more rapidly but at identical rates; and SCNMV-38 (sweet clover isolate), CRSV-A, and RCNMV-Aus moved fastest and slightly ahead of SCNMV-59 (alfalfa isolate). These variations can probably be explained by differences in the amino acid composition of the capsid proteins through mutation, possibly through adaptation to a new host, as can be suggested for the origin of the alfalfa isolate of SCNMV.

IV. MOLECULAR BIOLOGY

A. Pseudorecombinants

Pseudorecombination experiments allow the assignment of various characteristics of the dianthoviruses to the appropriate genome segment. Formation of pseudorecombinants was thought to be possible for any combination of RNA-1 and RNA-2 within and among the definitive dianthoviruses (Okuno *et al.*, 1983), but there is now one reported exception (Rao and Hiruki, 1987), which will be discussed below.

The aggregation and serological properties of pseudorecombinants of the A and N strains of CRSV (Dodds *et al.*, 1977) and the Can and Sw strains of RCNMV and SCNMV (Okuno *et al.*, 1983) demonstrated that the coat protein cistron is located on RNA-1. Dianthoviruses and the unrelated pea enation mosaic enamovirus (Chapter 12) are the only segmented genome plant viruses in which the coat protein gene is located on the larger genome segment.

Okuno *et al.* (1983) also studied the host response at 17 °C and 26 °C and serological properties of pseudorecombinants derived from homologous and heterologous mixtures of RNA species from SCNMV, RCNMV-Can, and RCNMV-Sw. The two RNA species in the pseudorecombinants maintained their original electrophoretic mobilities, which differed slightly among those of the parental viruses. Systemic infection of sweet clover at 26 °C was attributed to RNA-1 of SCNMV in pseudorecombinants of SCNMV and RCNMV-Sw. SCNMV, RCNMV-Can, and RCNMV-Sw did not infect white

clover at 26 °C, but pseudorecombinants containing RNA-1 of RCNMV-Can and RNA-2 of SCNMV (Can1:S2) or RNA-1 of RCNMV-Can and RNA-2 of RCNMV-Sw (Can1:Sw2) caused local infection in this host. White clover was not infected at either temperature by any pseudorecombinants containing SCNMV RNA-1. Pseudorecombinants S1:Can2 and Can1:S2 produced brownish rings on primary leaves of Red Kidney bean, a response that is intermediate between the whitish spots induced by RCNMV-Can and the rapid death following necrotic ringspots and veinal necrosis induced by SCNMV. These results clearly indicate that an interaction between RNA-1 and RNA-2 is involved in the type of host response to infection by dianthoviruses.

Lommel (1983) obtained an enhancement of RNA-1 infectivity with the pseudorecombinant of RCNMV RNA-1 and CRSV RNA-2 (R1:C2) but not with C1:R2. On serial passage in a systemic host (*Nicotiana clevelandii*) the yield of virus from R1:C2 gradually increased so that after 12 passages it was equal to those obtained from plants infected with the parental viruses. During these passages the electrophoretic mobility of the dsRNA of CRSV RNA-2 in R1:C2 gradually decreased. The recombinant nature of R1:C2 was established by cDNA hybridization, and further cDNA studies with S1 nuclease indicated small sequence differences between CRSV RNA-2 and the RNA-2 of the stabilized R1:C2. The host range of stabilized R1:C2, including the inability to infect carnation, was identical to that of RCNMV. However, the symptoms induced in *N. clevelandii* by R1:C2 differed from those of RCNMV and CRSV, suggesting an interaction between RNA-1 and RNA-2.

The exception to the general observation that pseudorecombination is possible between any two dianthoviruses occurred with RNA-1 of RCNMV-TpM48 and RNA-2 of RCNMV-TpM34 (Rao and Hiruki, 1987). When this mixture was inoculated to *Chenopodium quinoa*, no viral particles were detected by immunosorbent electron microscopy using antisera to each of the parental viruses, the infectivity of the RNA mixture was not significantly different from that of each RNA alone, and no viral RNA could be detected in the inoculated plants by dot blot nucleic acid hybridization, suggesting that replication of viral RNA did not occur. Moreover, Northern hybridization analysis indicated that cDNA probes to unfractionated RNA of each virus hybridized slightly with the heterologous virus at 42 °C but only with the homologous viral RNA at 55 °C. These results, coupled with the facts that RCNMV-TpM34 and RCNMV-TpM48 belong to serotypes A and B, respectively (Musil and Gallo, 1982), and that RCNMV-Can, which also belongs to serotype B, forms pseudorecombinants with RCNMV-Sw and SCNMV, suggest that RCNMV-TpM48 is distinctly different from other RCNMV strains and that its classification as a strain of RCNMV, rather than as a separate dianthovirus, should be reconsidered. This suggestion must be assessed in light of the fact that the isolates of RCNMV-TpM34 and RCNMV-Tp48 used by Rao and Hiruki (1987) differed from those used by earlier workers in being able to infect, respectively, cowpea and *Nicotiana*

clevelandii. Possibly these isolates represent host range mutants that retained their serological specificity but have an altered movement protein that is not expressed or, if expressed, is not functional in these hosts.

B. dsRNA Species

dsRNA species are involved in the replication of ssRNA plant viruses and are also useful adjuncts in the characterization of RNA viruses and in their diagnosis (Dodds *et al.*, 1984). Two major dsRNA species and a minor one were detected by sucrose density gradient centrifugation of extracts from cowpea (*Vigna unguiculata*) infected with CRSV strains A and N (Dodds *et al.*, 1977) and in bean infected with several isolates of RCNMV (Osman and Buck, 1991a). They were not detected in extracts from virus-free plants, indicating that they were specific RNA species induced by dianthovirus infection. The sizes of the two largest CRSV dsRNA species (A, B) suggested that they corresponded to the two genomic ssRNA species, and this was confirmed by the finding that, after denaturation at high temperature, they migrated at the same rate as the corresponding genomic ssRNA species. However, the relationship of the smallest RNA to the other two could not be immediately established. The mobility of the largest dsRNA (A) of RCNMV was similar for all isolates, and its estimated size (4.4 kbp) suggested that it corresponded to genomic RNA-1. The mobility of dsRNA-B was also uniform, with an estimated size of 1.4 kbp, but that of dsRNA-C was 1.2–1.3 kbp, depending on the virus isolate. Subsequent Northern blot analysis, using cDNA to RNA-1 plus RNA-2, or cDNA to RNA-1 only, indicated that (1) dsRNA-A was derived from RNA-1, (2) dsRNA-B corresponds to a subgenomic RNA derived from RNA-1, and (3) dsRNA-C is the dsRNA form of RNA-2. Lommel (1983) found that the dsRNA counterpart of genomic CRSV RNA-1 migrated faster than that of RCNMV, but the dsRNA-2 of CRSV migrated slower than that of RCNMV. This migration pattern of dsRNA paralleled differences in migration of the genomic RNA species of CRSV and RCNMV in nondenaturing gels. These results indicate that the genomes of definitive dianthoviruses are similarly organized and that they are distinct from those of other viruses (Morris, 1983).

C. Genome Strategy and Gene Function

1. Genome Organization

The dianthovirus genome consists of two distinct, positive-sense RNA species of about 4.5 kb (RNA-1) and 1.5 kb (RNA-2). The RNA-1 molecules of different dianthoviruses are similar in length [3756 bases for CRSV-type, 3889 for RCNMV-Aus, and 3876 for SCNMV-59 (alfalfa isolate)]; the RNA-2 molecules have lengths of 1394 bases for CRSV, 1448 for RCNMV-Aus, and

1446 and 1449 for SCNMV-38 (sweet clover isolate) and SCNMV-59, respectively. The complete nucleotide sequence of each of these genomes has been published (Ryabov *et al.*, 1994; Kendall and Lommel, 1992; Xiong and Lommel, 1989; Osman *et al.*, 1991a; Ge *et al.*, 1992, 1993). The genomic organization of RCNMV is shown in Fig. 2. Those of CRSV and SCNMV are similar and so probably is that of FNSV, although its genome has not been sequenced.

The 5′ terminus of each genomic RNA species of RCNMV has a m^7GpppA cap (Xiong and Lommel, 1989) and it is likely that those of the other dianthoviruses are similarly capped. The 3′ termini of the genomic RCNMV RNA species and the other definitive dianthoviruses are not polyadenylated, but each has a nearly identical sequence of 27 nucleotides (Xiong and Lommel, 1989) that is capable of forming a stable stem-loop structure

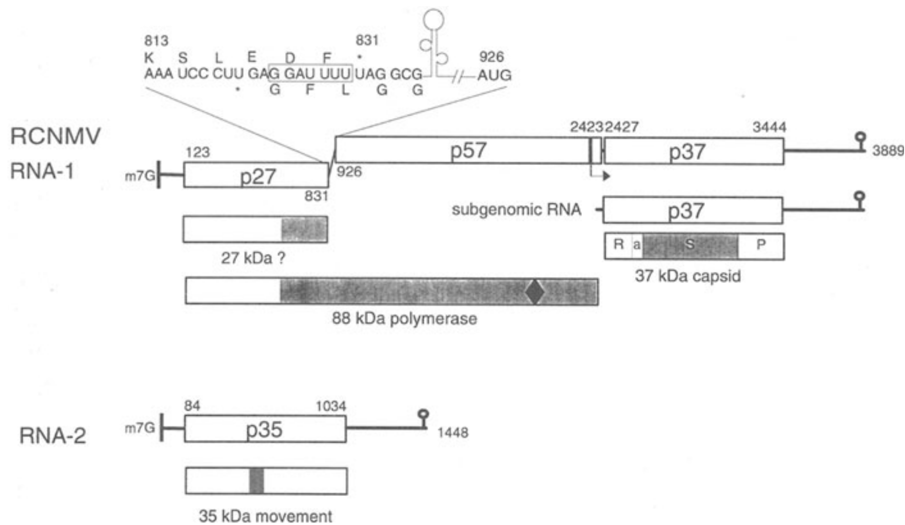

FIGURE 2. Organization and expression of the RCNMV genome. Untranslated regions in RCNMV RNA-1 and RNA-2 are depicted as solid lines and the ORFs are depicted as open rectangles. Vertical bars at the 5′ ends of RNA-1 and RNA-2 and near the 3′ terminus of the p57 ORF identify a 13-nucleotide conserved sequence. The right-angle arrow identifies the probable location of the beginning of the capsid protein subgenomic RNA. The stem-loop at the 3′ end of both viral RNA species represents the 3-terminal 27 homologous nucleotides that are capable of forming a stable stem-loop structure. The diagonal line identifies the region of ribosomal frameshift, allowing readthrough of the p27 ORF. Rectangles below the RNA species represent virus-encoded polypeptides. The shaded areas in the proteins encoded by RNA-1 identify domains of significant amino acid sequence similarity to counterparts in the carmoviruses, necroviruses, machlomoviruses, and tombusviruses. The diamond-shaped marker in the shaded region of the 88-kDa protein represents conserved polymerase motifs. The rectangles identified by R, a, S, and P in the 37-kDa capsid protein encoded by the subgenomic RNA of RNA-1 represent the random (RNA-binding), arm, shell, and protruding domains, respectively. The shaded region in the movement protein encoded by RNA-2 identifies a motif that is conserved in the dianthoviruses and bromoviruses. (After Giesman-Cookmeyer *et al.*, 1995.)

thought to be involved in recognition of the viral RNA replicase and in the initiation of the synthesis of (−)-strand RNA. The existence of this conserved sequence at the 3′ ends of both RCNMV RNA species, coupled with the fact that pseudorecombinants can be formed by mixing RNA-1 and RNA-2 from different dianthoviruses, suggest that there are conserved sequences in the 3′ ends of both RNA species of all dianthoviruses.

Three large open reading frames (ORF) have been identified in RNA-1 of the definitive dianthoviruses: a 5′-terminal ORF encoding a polypeptide of 27 kDa; an internal one encoding a polypeptide of 54–57 kDa (57 kDa in Fig. 2); and a 3′-terminal one that encodes the 37- to 39-kDa capsid protein (37 kDa in Fig. 2). A heptanucleotide sequence located immediately before the 27-kDa termination signal in RCNMV RNA-1 is followed by a 95-nucleotide intergenic region between the 27-kDa and 57-kDa ORF; this intergenic region contains a stable stem-loop structure (Xiong *et al.*, 1993b). These two stretches of sequence, first recognized in the *gag-pol* region of eukaryotic retroviruses (Varmus, 1988), are both essential to a ribosomal frameshifting event involved in the synthesis of the 88-kDa viral RNA polymerase (Kim and Lommel, 1994). Similar structures have been identified in RNA-1 of CRSV and SCNMV (Ryabov *et al.*, 1994) and others are thought to be similarly involved in the synthesis of the RNA polymerases of barley yellow dwarf (Veidt *et al.*, 1988) and potato leafroll luteoviruses (Prüfer *et al.*, 1992) and other plus-strand RNA viruses (Atkins *et al.*, 1990).

CRSV RNA-1 has a putative ORF, not detected in RNA-1 of the other dianthoviruses, downstream from the ORF for the capsid protein, and this ORF could encode a polypeptide of 10 kDa (Ryabov *et al.*, 1994). However, because it is followed by a noncoding region of only nine nucleotides, this ORF may not be expressed. Another possibility is that the amber termination codon for the 38-kDa capsid protein ORF is suppressed during translation, in which case a 51-kDa read-through protein composed of the capsid protein and the putative 10-kDa polypeptide would be synthesized.

Dianthovirus RNA-2 molecules appear to be monocistronic, encoding polypeptides of 33.8–36.5 kDa (35 kDa in Fig. 2).

2. Functions of Gene Products

Functions have been assigned to the major proteins encoded by all the dianthovirus ORFs with the exception of the putative 10-kDa polypeptide of CRSV. Results from pseudorecombination studies among the definitive dianthoviruses, coupled with the observation that the RCNMV RNA-1 molecule replicates in plant protoplasts and induces the production of typical virus particles (Osman and Buck, 1987), indicate that the genes for RNA polymerase and the capsid protein are located on RNA-1 (Dodds *et al.*, 1977; Okuno *et al.*, 1983). Similar conclusions were reached following *in vitro* translation of the genomic RNA species of RCNMV (Morris-Krsinich *et al.*, 1983; Lommel, 1983) and CRSV (J. H. Tremaine, unpublished results). Both

RNA species are required for systemic infection of plants (Gould *et al.*, 1981; Okuno *et al.*, 1983; Osman *et al.*, 1986; Paje-Manalo and Lommel, 1989), suggesting that the 35-kDa protein encoded by RNA-2 is the transport or movement protein involved in cell-to-cell movement and systemic infection of plants by dianthoviruses. Neither of the RCNMV genomic segments replicated independently in inoculated leaves of *Nicotiana clevelandii* (Paje-Manalo and Lommel, 1989) nor did those of SCNMV in cowpea (*Vigna unguiculata*) (Hiruki *et al.*, 1992). These results do not constitute proof that RNA-1 did not replicate in inoculated leaf cells because the low efficiency of mechanical inoculation would make it difficult to detect viral protein or RNA in the few cells infected. Moreover, RNA-1 would probably not be able to move from inoculated cells to adjoining cells in the absence of the movement protein provided by RNA-2.

Experiments in which infective RNA-1 transcripts of cloned DNA from capsid protein mutants, which lacked portions of the capsid protein gene at the 3′ terminus, were co-inoculated with wild-type RNA-2 showed that the capsid protein gene is not required for cell-to-cell spread within inoculated leaves of *N. benthamiana* at 15 °C or 25 °C. Systemic spread did occur, however, at the lower but not the higher temperature (Xiong *et al.*, 1993a). There were no differences between wild type and mutants in the type of symptom or its time of appearance in inoculated leaves. Although these mutant capsid proteins were synthesized and stable *in vitro*, no antigens corresponding to their predicted sizes were detected by Western blotting of extracts from inoculated or noninoculated leaves infected by these mutants, indicating that the mutant proteins were probably unstable and did not accumulate in infected plants. Neither *N. benthamiana* nor *N. clevelandii* were systemically infected at 25 °C, although occasional "oak-leaf" symptoms were observed at 4 weeks postinoculation in leaves of *N. benthamiana* immediately above the inoculated leaves, probably as a result of slow cell-to-cell spread through the stem. These results are interesting because *N. benthamiana* is a host for a number of viruses that do not infect other *Nicotiana* species. In these instances, *N. benthamiana* may be unusually permissive to rapid intercellular movement of such viruses. The results also clearly indicate that movement of RCNMV, and presumably that of other dianthoviruses, is dependent on the interaction of viral and host genetic factors and environmental conditions.

Polypeptide products of *in vitro* translation of RCNMV RNA-1 in a rabbit reticulocyte translation system corresponded to the predicted molecular mass of each ORF (Xiong and Lommel, 1989) with the exception of a 90-kDa polypeptide that was produced in very low amounts and did not correspond to any ORF. This was considered to be probably a read-through product of the ORFs for the 27-kDa and 57-kDa polypeptides. Two polyclonal antisera to synthetic oligopeptides, one representing the C-terminal portion of the 27-kDa polypeptide and the other representing the C-terminal region of the 57-kDa polypeptide, and therefore the C-terminal region of the putative

88-kDa read-through protein, precipitated not only their homologous polypeptides but also the 88-kDa polypeptide (Xiong *et al.*, 1993b), providing evidence that it is indeed a read-through protein. Although the 57-kDa polypeptide encoded in RNA-1 of RCNMV is produced *in vitro*, it could not be detected *in vivo* using an antibody to the C terminus of the 57/88-kDa polypeptide or antibody to a fusion protein including the 57-kDa protein (Kim and Lommel, 1994). Infective RNA transcripts of mutants that lack the ORF initiation codon for the 57-kDa protein or a downstream, in-frame methionine codon were unable to synthesize the protein *in vitro*, but they were indistinguishable from wild type in their ability to infect plants systemically (Kim and Lommel, 1994), suggesting that the 57-kDa polypeptide is an artifact of *in vitro* translation or, if produced *in vivo*, is not needed for systemic spread. The 88-kDa polypeptide contains conserved RNA-dependent RNA polymerase motifs, suggesting that it is the viral RNA polymerase (Koonin, 1991). The heptanucleotide sequence that facilitates ribosomal frameshifting was identified in RNA-1 immediately behind the amber termination codon for the 27-kDa ORF, and point and frameshift mutations within this sequence confirmed its involvement in *in vitro* synthesis of the 88-kDa polypeptide (Xiong *et al.*, 1993b). It is likely that the putative viral RNA polymerase is expressed *in vivo* by a −1 ribosomal frameshifting mechanism similar to that of coronaviruses (Brierley *et al.*, 1989) and retroviruses (Varmus, 1988). The 37-kDa capsid protein is expressed via a 3′-coterminal subgenomic RNA of 1.5 kb.

The RNA-2 species of dianthoviruses (Kendall and Lommel, 1992; Ge *et al.*, 1992; Lommel *et al.*, 1988; Osman *et al.*, 1986) is required for systemic infection of plants, suggesting that its 35-kDa protein product is the movement protein (MP) for dianthoviruses. Deletion mutants, lacking no more than 39 amino acids from the C-terminal end of the MP, moved as rapidly as wild type and induced typical symptoms on inoculated and systemically infected leaves in *N. benthamiana*, but mutants with larger deletions failed to move from cell to cell or systemically in this host (Xiong *et al.*, 1993a). The failure of a spontaneous mutant of RCNMV-TpM34, which induced necrotic rather than chlorotic local lesions in cowpea, to infect this host systemically was attributed to the deletion of one of a sequence of four A residues between nucleotides 790 and 793 of RNA-2 (Osman *et al.*, 1991b). Infective RNA transcripts of cloned DNA of the mutant RNA-2 that contained an inserted A residue in the sequence restored the ability of the combination of mutant RNA-2 and wild-type or mutant RNA-1 to infect cowpea systemically. These results clearly prove that the 35-kDa protein controls cell-to-cell spread of dianthoviruses. The dianthovirus MP appears to be analogous in function to that of the tobamoviruses which accumulates in the plasmodesmata and modifies them to allow passage of molecules larger than the normal solutes (Lucas and Wolf, 1993). Tobamovirus MP molecules also bind ssRNA *in vitro*, and presumably *in vivo*, and it is thought that these MP–viral RNA complexes are the form in which some viruses move intercellularly in plants

(Hull, 1989; Citovsky *et al.*, 1992). RCNMV MP, which accumulates in cell wall fractions (Osman and Buck, 1991b) and perhaps in plasmodesmata, also binds ssRNA transcripts corresponding to the 5′-terminal nucleotides of the RCNMV MP gene (Osman *et al.*, 1992; Giesman-Cookmeyer and Lommel, 1993), and MP mutants derived by alanine-scanning mutagenesis differed in the symptoms they induced and in their ability to spread intercellularly (Giesman-Cookmeyer and Lommel, 1993). Studies of RCNMV MP mutants enabled three MP domains to be identified. Some mutant MPs differed in their capacity to bind ssRNA; others differed in the degree of cooperativity of such binding; and a third class did not depend on these properties. In further studies (Fujiwara *et al.*, 1993), fluorescein-labeled dextran, co-injected into leaf mesophyll cells of cowpea with either wild-type RCNMV MP or movement-effective mutant MP, moved rapidly to adjoining cells but failed to move when injected alone or with movement-defective mutant MP. Similar results were obtained in experiments with labeled wild-type and movement-effective mutant MP or movement-defective mutant MP. These results strongly suggest that the RCNMV MP and viral RNA–MP complexes move via plasmodesmata.

D. Sequence Relationships

1. Among Dianthoviruses

The dianthoviruses have a very high degree of nucleotide and amino acid sequence similarity. The overall homology between the RNA-1 species of RCNMV-Aus and SCNMV is 80%, but when comparison is confined to their respective RNA polymerases, there is over 90% homology at the amino acid level, clearly suggesting the presence of highly conserved sequences in this polypeptide. Similar analyses indicate that the preframeshift 27-kDa protein of CRSV shares 71 and 63% amino acid sequence identity with the comparable proteins of RCNMV and SCNMV, respectively, and the CRSV 57-kDa polypeptide shares 79 and 76% sequence identity with the comparable proteins of RCNMV and SCNMV, respectively (Ryabov *et al.*, 1994). The least degree of homology was among the capsid proteins: about 74% between the aligned sequences of RCNMV and SCNMV (Ge *et al.*, 1993) but only 51–54% between those of CRSV and either RCNMV or SCNMV (Ryabov *et al.*, 1994). Assuming that the three-dimensional structure of the CRSV capsid is similar to that of turnip crinkle tombusvirus, as determined by X-ray crystallography (Dolja and Koonin, 1991; Carrington *et al.*, 1987), the protruding domain of the capsid protein is much less conserved (34%) than the shell domain (58%).

Comparison of the nucleotides of the RNA-2 species of RCNMV and SCNMV indicates that they too are similar in organization. The RNA-2 species of RCNMV-Aus (Lommel *et al.*, 1988) and RCNMV-TpM34 (Osman

et al., 1991a) from Czechoslovakia (each of 1448 nucleotides) have considerable nucleotide sequence similarity with the two SCNMV strains (84.2 and 94.8%, respectively, with SCNMV-38, and 82.7 and 94.3%, respectively, with SCNMV-59) (Ge *et al.*, 1992). Comparison of the MPs encoded by these RNA-2 species indicates that the two SCNMV strains are more closely related to RCNMV-TpM34 than to RCNMV-Aus. The 34-kDa MP encoded by CRSV RNA-2 has 59.6 and 55.7% amino acid sequence identity with the corresponding proteins of RCNMV-Aus and RCNMV-TpM34, respectively. The N-terminal 230 amino acids are even more highly conserved, with sequence identities of 64.3 and 62.6%, respectively (Kendall and Lommel, 1992). These comparisons suggest that the organization of the RNA-2 species and the functional domains of the encoded protein may be similar among the dianthoviruses.

2. Between Dianthoviruses and Other Viruses

The *Dianthovirus* genus shares features with other genera of plant viruses. The 88-kDa RNA polymerase encoded in RNA-1 has a high degree of sequence similarity to those of the *Tombusvirus*, *Carmovirus*, *Luteovirus*, *Machlomovirus*, and *Necrovirus* genera (Koonin and Dolja, 1993). Moreover, the amino acid sequences of the shell (S) and protruding (P) domains of the capsid protein, which are also encoded in RNA-1, are highly and moderately conserved, respectively, with those of the *Tombusvirus* and *Carmovirus* genera (Rochon *et al.*, 1991). There is a small amount of amino acid sequence identity between the MP encoded in RCNMV-Aus and the 3a protein (MP) of the bromoviruses, brome mosaic (23.6% over a 54-amino acid sequence) (Lommel *et al.*, 1988) and cowpea chlorotic mottle (19.7% over a 157-amino acid sequence) (Allison *et al.*, 1989). A similar low amount of sequence identity with the 3a proteins of these bromoviruses was found in the corresponding protein of RCNMV-TpM34 (Osman *et al.*, 1991a). However, similar degrees of relatedness were found in many unrelated proteins (Osman *et al.*, 1991a), and it remains to be determined if there is any functional significance to the presence of these related sequences in the movement proteins of dianthoviruses and bromoviruses.

V. CYTOPATHOLOGY

There is a paucity of information on the cytopathology of dianthovirus infections. In CRSV-infected sweet william and cowpea, large crystalline arrays of virus particles occurred in the cytoplasm, and the nuclei contained large aggregates of virus particles as well as tubular inclusions, often with virus particles on their surfaces (Weintraub *et al.*, 1975). Spherical inclusion bodies were also seen in nuclei of sweet william but not in cowpea. Inflated, electron-transparent mitochondria, clusters of proliferated endoplasmic re-

ticulum with dilated cisternae, and scattered CRSV-like particles were common in the cytoplasm of CRSV-infected leaf mesophyll cells in *Chenopodium quinoa* and *Nicotiana megalosiphon* (Koenig *et al.*, 1988); necrotic cells contained massive aggregates of these particles. Viruslike particles were also observed in nuclei, but the tubular inclusions observed in this organelle by Weintraub *et al.* (1975) were not detected. RCNMV was present in leaves, stems, and roots of infected red clover (Hollings and Stone, 1977); membranous vesicles and patches of densely stained amorphous material were common in the cytoplasm (Francki *et al.*, 1985) and chloroplastic vesiculation was observed in red clover infected with RCNMV-Can (Ragetli and Elder, 1977). Amoeboid intracellular inclusions were seen in about 5% of the cells in an experimental host (*N. clevelandii*) but apparently not in red or white clover. Large amorphous inclusions seen by light microscopy in the cytoplasm of Red Kidney bean (*Phaseolus vulgaris*) infected with SCNMV were subsequently shown to be composed of large aggregates of virus particles when examined by electron microscopy (Hiruki *et al.*, 1984b). In fique infected with FNSV, viruslike particles were readily detected in root and leaf cells, often associated with large, electron-dense tubular inclusions (Morales *et al.*, 1992) and "aster" (flower)-like inclusions (Dabek and Castaño, 1978).

VI. ECOLOGY AND CONTROL

A. Ecology

Little is known about the survival and spread of dianthoviruses in nature. The viruses are not transmitted by aerial vectors so far tested, although there is an unconfirmed report that the aphid *Myzus persicae*, but not the scale insect *Saissetia caffea* or the mealy bug *Planococcus citri*, can transmit FNSV, albeit inefficiently (Dabek and Castaño, 1978). Given the highly contagious nature of the dianthoviruses, such transmission may be the result of contamination rather than of true vector transmission. None of the dianthoviruses is known to be transmitted by seed. Despite the fact that pollen from SCNMV-infected sweet clover is heavily contaminated with surface-borne virus and is constantly present on the western flower thrips (*Frankliniella occidentalis*), which inhabit florets of sweet clover, no transmission of SCNMV was observed in several controlled experiments (Hiruki *et al.*, 1989). Rather, contact transmission as well as a soil-borne mode of transmission, without the aid of vectors, are clearly indicated by experimental evidence. Moreover, the natural host range of CRSV includes species in which the virus can be spread by vegetative propagation.

Of the three routes of spread, that in the soil is of current interest because of the controversial evidence for the role of soil-inhabiting organisms as vectors. Susceptible plants, transplanted into soil that previously contained infected plants or into sterilized soil to which infected plant

tissues or virus suspensions had been added, were readily infected (Bowen and Plumb, 1979; Brown and Trudgill, 1984; Gerhardson and Insunza, 1979; Hollings and Stone, 1977; Kegler and Kegler, 1981; Lyness and Teakle, 1981). Particles of CRSV, RCNMV, and SCNMV are released from root cells into soil water (Hollings and Stone, 1977; Kegler and Kegler, 1981; Hiruki, 1986a) where they may retain infectivity for months. Presumably plants in the natural state can be inoculated by infective virions in the soil solution. Claims that the dianthoviruses are transmitted by longidorid nematodes (Fritzsche, 1968; Fritzsche and Schmelzer, 1967; Fritzsche *et al.*, 1979; Kleinhempel *et al.*, 1980) or by species of the Chytridiomycete, *Olpidium* (Lange, 1977; MacFarlane, 1981) have not been confirmed (Brown and Trudgill, 1984; Lyness and Teakle, 1981). The two last-named groups of investigators demonstrated soil transmission of CRSV or RCNMV to test plants in soil known not to contain the putative vectors. Transmission of CRSV to test plants in the presence of nematodes (*Longidorus elongatus*) was no greater than in their absence (Brown and Trudgill, 1984), but transmission of RCNMV to test plants was enhanced 2- to 20-fold in the presence of *O. brassicae* (Gerhardson and Insunza, 1979), suggesting perhaps that the fungus may play a role in initiating RCNMV infection. However, in controlled inoculations, the tobacco isolate of *O. brassicae* was not found in the roots of SCNMV-infected sweet clover seedlings nor was there a significant difference in the percentage of sweet clover seedlings infected by inocula that contained zoospores of *O. brassicae* and those that did not (Hiruki, 1986a). MacFarlane (1982) reported that zoospores of *O. bornovanus* (Sahtiyanci) Karling [= *O. radicale* Schwartz & Cook fide Lange & Insunza; = *O. cucurbitacearum* Barr & Dias (Campbell and Sim, 1994)], probably more widespread in Britain than previously recognized, transmitted RCNMV to clover, leading to the tentative conclusion that the fungus is a vector. Clearly, further work is needed to determine if this or any other fungus is a vector of the virus or a factor in predisposing host plants to root infection.

An important consideration in all of the studies in which putative vectors are collected from the roots of infected plants is the possibility of contamination with infective virus particles present in soil water or on the root surface. The presence of infective dianthovirus particles in soil water and the isolation of CRSV from a pond surrounded only by grassland, from a stream that had no direct contact with agricultural fields (Yi *et al.*, 1992), and from a canal near a sewage plant (Koenig *et al.*, 1988) parallel reports of the isolation from river waters of other plant viruses such as an unidentified sugar beet virus (Tomlinson *et al.*, 1983a), tobamoviruses (Koenig, 1986, 1988; Tosic and Tosic, 1984), necroviruses (Tomlinson *et al.*, 1983b), potexviruses and carnation mottle virus (Koenig and Lesemann, 1985), and especially tombusviruses (Tomlinson and Faithfull, 1984; Koenig and Lesemann, 1985; Koenig *et al.*, 1989). Thus, both for dianthoviruses and for tombusviruses, transport by water affords a distribution system that effectively contributes to their spread. Isolation of CRSV from a sewage canal does not necessarily

mean that the virus was originally present in sewage, but it would be interesting to determine if dianthoviruses remain infective after passage through the alimentary tracts of livestock (and migratory animals), as has been reported for TBSV in human feces (Tomlinson *et al.*, 1982) and for several plant viruses including TBSV in the feces of rabbits and/or mice (Kegler *et al.*, 1984). If so, an additional means of spread (e.g., in manure for fertilizer) is possible.

B. Control

Aside from the usual recommendation that proper sanitation be employed in plant propagation, the choice of additional control measures is dictated by the affected species. In vegetatively propagated plants such as carnation and the tree fruits, eradication of viruses in plant shoots and meristems by thermotherapy (Brierley, 1964) or meristem-tip culture (Stone, 1968; Kowalska, 1974) can be used to produce virus-free nuclear stocks as the basis for commercial propagation. Propagation from such stocks, coupled with adequate monitoring by suitable methods such as ELISA (Lommel *et al.*, 1983), should ensure continued production of virus-free plants. In California (Lommel *et al.*, 1983), and presumably in the rest of North America, CRSV infection of commercial carnation plantings is no longer of significance, but the virus has been reported occasionally in carnation in other parts of the world (Bennet and Milne, 1976; Bremer and Lahdenpera, 1981; Valenzuela and Pizano, 1992). Carnation and sweet william are probably the only herbaceous hosts of concern with respect to CRSV infection, but the role of CRSV in the etiology of disease in pome and stone fruits requires further study.

Control of RCNMV, especially because of its broader natural host range, would appear to be more difficult than that of CRSV. Because RCNMV is not seed-transmitted, seedlings of clovers and alfalfa presumably become infected by contact transmission or by soil-borne virus. Unfortunately, most of the red clover cultivars recommended for production in Britain are susceptible to natural infection by RCNMV, some (Hungoropoly and Teroba) showing about 45% natural infection (Bowen and Plumb, 1979). Similar results were obtained following mechanical inoculation of red and white clover cultivars commonly grown in Czechoslovakia (Musil *et al.*, 1979). The stability of RCNMV infectivity in soil would appear to preclude soil treatment as a method of eradicating the virus. However, planting a mixture of Hungoropoly clover (5 kg/hectare) and Italian ryegrass cv RvP (13 kg/hectare) resulted in 0.8% RCNMV infection compared to 8.9% for a pure stand of Hungoropoly (Lewis *et al.*, 1985), suggesting that a mixture of susceptible and immune crop species may significantly reduce the incidence of infection. However, genetic resistance or tolerance to RCNMV infection should be investigated for long-term disease control.

VII. CONCLUDING REMARKS

Although the experimental host ranges of the dianthoviruses are very wide, the natural host ranges are limited to a few species. Application of the rapid and sensitive assay and identification procedures currently available for these viruses may result in their detection in other crops and weeds. The absence of symptoms in RCNMV-infected plants at higher temperatures may explain why more such reports have not been made to date.

Pseudorecombinants between definitive dianthoviruses have been produced experimentally, suggesting that genetic reassortment between these viruses could possibly occur under field conditions if a common host were present. There is no evidence for this, possibly because two dianthoviruses have not been found in the same host in the same place. In Canada, RCNMV was isolated from a single red clover plant near Vancouver (Ragetli and Elder, 1977), and in a systematic search in Alberta, C. Hiruki (unpublished results) failed to detect red clover, white clover, or alsike clover infected with RCNMV. Interestingly, however, the natural hosts of RCNMV include alfalfa and white clover in Australia (Gould *et al.*, 1981; Lyness and Teakle, 1981) and sweet clover in Europe (Musil, 1969a). Perhaps a host plant such as alfalfa, which is naturally susceptible to RCNMV and SCNMV in single infections, would be susceptible to both viruses in mixed infections. If natural infection of the roots of field plants is via water-borne virus in soil, the opportunities for mixed infections with both RCNMV and SCNMV might be significant.

Further research is required to characterize the early events of virus infection as it occurs in nature, i.e., in roots apparently in the absence of a vector. Are there intrinsic properties of the dianthovirus virion that result in infection of root cells under conditions in which other plant viruses do not infect? The soil-borne nature and similar infection pattern of the dianthoviruses and tombusviruses, coupled with similarities in the primary sequences of the amino acids in their capsid proteins, suggest that common features may exist in the early stages of the interaction between these viruses and plant roots. Although the evidence for dianthovirus transmission by soil-inhabiting fungi is equivocal, cucumber necrosis tombusvirus (CuNV) is naturally transmitted by zoospores of *O. bornovanus*, and a chimeric virus, containing the genome of TBSV modified by substitution of the capsid gene of TBSV by that of CuNV, was transmitted by *O. bornovanus* to *Nicotiana clevelandii* (McLean *et al.*, 1994). The reciprocal chimeric virus was not transmitted by the fungus. CuNV probably diverged from an ancestral virus in its evolution and acquired genes for specific receptor sites, probably located in the protruding domain, which allowed specific recognition of the virus by *O. bornovanus* and its subsequent transmission.

A second aspect of the basic interaction of dianthoviruses and plant root systems is the route of distribution of viruses from the initially infected root cells. Does the dianthovirus MP play any role in this process? Is the ORF for

the MP translated in the roots of "nonhosts" (Paje-Manalo and Lommel, 1989), the leaf protoplasts of which support replication of RCNMV RNA-1? If the MP is expressed in "nonhost" plants, does it interact with the plasmodesmata as it does in susceptible hosts? Evidence is accumulating that indicates that viral MPs play a significant role in the host range of plant viruses (Mise *et al.*, 1993) and the availability of infective RNA transcripts for RCNMV (Xiong and Lommel, 1991) and SCNMV (Ge and Hiruki, 1993) provides a unique opportunity for research on genetic manipulation of MP genes and their subsequent interaction in different plant genotypes.

Utilization of plants transformed with dianthovirus genes should allow continued progress in the research on basic aspects of the interaction between viral and host genes that governs the responses of plants to infection by dianthoviruses. The physicochemical and biological properties of the dianthoviruses make them eminently suitable tools for future research on the basic aspects of disease induction by plant viruses.

REFERENCES

Allison, R. F., Janda, M., and Ahlquist, P., 1989, Sequence of cowpea chlorotic mottle virus RNAs 2 and 3 and evidence of a recombination event during bromovirus evolution, *Virology* **172:**321.

Altschuh, D., and Van Regenmortel, M. H. V., 1983, Refutation of the alleged antigenic relationship between an isometric and a rod shaped plant virus, *Int. J. Microbiol.* **1:**13.

Atkins, J. F., Weiss, R. B., and Gesteland, R. F., 1990, Ribosome gymnastics—degree of difficulty 9.5, style 10.0, *Cell* **62:**413.

Barton, R. J., 1981, Analysis of virus capsid polypeptides, *Rep. Glasshouse Crops Res. Inst. 1980:*151.

Bennet, P. R., and Milne, K. S., 1976, Carnation viruses in New Zealand, *Acta. Hortic.* **59:**61.

Bercks, R., and Querfurth, G., 1972, Serologische Beziehungen von cocksfoot mild mosaic virus und carnation ringspot virus zu Viren der turnip yellow mosaic virus-Gruppe, *Phytopathol. Z.* **75:**215.

Bowen, R., and Plumb, R. T., 1979, The occurrence and effects of red clover necrotic mosaic virus in red clover (*Trifolium pratense*), *Ann. Appl. Biol.* **91:**227.

Bremer, K., and Lahdenpera, M.-L., 1981, Virus diseases in carnation and chrysanthemum cuttings imported into Finland, *Ann. Agriculturae Fenniae* **20:**214.

Brierley, I., Digard, P., and Inglis, S. C., 1989, Characterization of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot, *Cell* **57:**548.

Brierley, P., 1964, Heat cure of carnation viruses, *Plant Dis. Rep.* **48:**143.

Brown, D. J. F., and Trudgill, D. L., 1984, The spread of carnation ringspot virus in soil with or without nematodes, *Nematologica* **30:**102.

Campbell, R. N., and Sim, S. T., 1994, Host specificity and nomenclature of *Olpidium bornovanus* (= *Olpidium radicale*) and comparisons to *O. brassicae*, *Can. J. Bot.* **72:**1136.

Carrington, J. C., Morris, T. J., Stockley, P. G., and Harrison, S. C., 1987, Structure and assembly of turnip crinkle virus. IV. Analysis of the coat protein gene and implications of the subunit primary structure, *J. Mol. Biol.* **194:**265.

Casper, R., 1976, Natural occurrence of cucumber mosaic virus with plum pox virus (Sharka) and prunus necrotic ringspot virus in plum, *Mitt. Biol. Bundesanst. Land-u.Forstwirtsch.* **170:**33.

Citovsky, V., Wong, M.-L., Shaw, A. L., Venkataram Prasad, B. V., and Zambryski, P., 1992, Visualization and characterization of tobacco mosaic virus movement protein binding to single-stranded nucleic acids, *Plant Cell* **4**:397.

Dabek, A. J., and Castaño, J. J., 1978, The occurrence, symptomatology, transmission and virus aetiology of macana disease of fique (*Furcraea* spp.) in Colombia, South America, *Phytopathol. Z.* **92**:57.

Dodds, J. A., Tremaine, J. H., and Ronald, W. P., 1977, Some properties of carnation ringspot virus single- and double- stranded ribonucleic acid, *Virology* **83**:322.

Dodds, J. A., Morris, T. J., and Jordan, R. L., 1984, Plant viral double-stranded RNA, *Annu. Rev. Phytopathol.* **22**:151.

Dolja, V. V., and Koonin, D. V., 1991, Phylogeny of capsid proteins of small icosahedral RNA plant viruses, *J. Gen. Virol.* **72**:1481.

Francki, R. I. B., Milne, R. G., and Hatta, T., 1985, *Atlas of Plant Viruses*, Vol. II, CRC Press, Boca Raton, FL.

Francki, R. I. B., Fauquet, C. M., Knudson, D. L., and Brown, F. (eds.), 1991, *Classification and Nomenclature of Viruses: Fifth Report of the International Committee on Taxonomy of Viruses*, Springer-Verlag, Vienna (also in *Arch. Virol.* Suppl. 2).

Fritzsche, R., 1968, Ökologie und Vektoreignung von *Longidorus macrosoma* Hooper, *Biol. Zentralbl.* **87**:139.

Fritzsche R., and Schmelzer, K., 1967, Übertragbarkeit des Nelkenringflecken Virus durch Nematoden, *Naturwissenschaften* **54**:498.

Fritzsche, R., Kegler, H., Thiele, S., and Gruber, G., 1979, Beitrag zur Epidemiologie und Übertragung des Nelkenringflecken–Virus in Obstbestanden, *Arch. Phytopathol. Pflanzenschutz* **15**:177.

Fujiwara, T., Giesman-Cookmeyer, D., Ding, B., Lommel, S. A., and Lucas, W. J., 1993, Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the red clover necrotic mosaic virus movement protein, *Plant Cell* **5**:1783.

Gallo, J., and Musil, M., 1984a, Serotypes of red clover necrotic mosaic virus. III. Immunoelectrophoresis under different conditions, *Acta Virol.* **28**:78.

Gallo, J., and Musil, M., 1984b, Isoelectric points of red clover necrotic mosaic virus serotypes, *Acta Virol.* **28**:82.

Ge, Z., and Hiruki, C., 1993, The infectious transcripts of sweet clover necrotic mosaic virus bipartite genome constructed by the polymerase chain reaction, *Proc. Japan Acad. Ser. B* **69**:113.

Ge, Z., Hiruki, C., and Roy, K. L., 1992, A comparative study of the RNA-2 nucleotide sequences of two sweet clover necrotic mosaic virus strains, *J. Gen. Virol.* **73**:2483.

Ge, Z., Hiruki, C., and Roy, K. L., 1993, Nucleotide sequence of sweet clover necrotic mosaic dianthovirus RNA-1, *Virus Res.* **28**:113.

Gerhardson, B., and Insunza, V., 1979, Soil transmission of red clover necrotic mosaic virus, *Phytopathol. Z.* **94**:67.

Gerhardson, B., and Lindsten, K., 1973, Red clover mottle virus and red clover necrotic mosaic virus in Sweden, *Phytopathol. Z.* **76**:67.

Giesman-Cookmeyer, D., and Lommel, S. A., 1993, Alanine scanning mutagenesis of a plant virus movement protein identifies three functional domains, *Plant Cell* **5**:973.

Giesman-Cookmeyer, D., Kim, K.-H., and Lommel, S. A., 1995, Dianthoviruses, in: *Pathogenesis and Host-Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Basis*. Vol. 3: *Viruses and Viroids* (R. P. Singh, U. S. Singh, and K. Kohmoto, eds.), Pergamon Press, Oxford pp. 157–176.

Gould, A. R., Francki, R. I. B., Hatta, T., and Hollings, M., 1981, The bipartite genome of red clover necrotic mosaic virus, *Virology* **108**:499.

Hatta, T., and Francki, R. I. B., 1984, Differences in the morphology of isometric particles of some plant viruses stained with uranyl acetate as an aid to their identification, *J. Virol. Methods* **9**:237.

Hiruki, C., 1986a, Incidence and geographic distribution of sweet clover necrotic mosaic virus in Alberta, *Plant Dis.* **70**:1129.

Hiruki, C., 1986b, Sweet clover necrotic mosaic virus, *AAB Descriptions of Plant Viruses* No. 321.

Hiruki, C., 1987, The dianthoviruses: A distinct group of isometric plant viruses with bipartite genome, *Adv. Virus Res.* **33**:257.

Hiruki, C., and Figueiredo, G., 1985, Monoclonal antibodies and their use in the study of dianthoviruses, *Acta Hortic.* **164**:217.

Hiruki, C., Rao, A. L. N., Furuya, Y, and Figueiredo, G., 1984a, Serological studies of dianthoviruses using monoclonal and polyclonal antibodies, *J. Gen. Virol.* **65**:2273.

Hiruki, C., Rao, D. V., Chen, M. H., Okuno, T., and Figueiredo, G., 1984b, Characterization of sweet clover necrotic mosaic virus, *Phytopathology* **74**:482.

Hiruki, C., Kudo, K., and Figueiredo, G., 1989, Transmission of sweet clover necrotic mosaic virus, *Proc. Japan Acad. Ser. B* **65**:234.

Hiruki, C., Kakuta, H., Ge, Z., Figueiredo, G., and Mizutani, J., 1992, Viral genome delivery into detached and intact leaf tissues of *Vigna unguiculata* by RNA-coated gold particles using the improved particle gun, *Proc. Japan Acad. Ser B* **68**:183.

Hollings, M., and Stone, O. M., 1965, Investigations of carnation viruses. II. Carnation ringspot, *Ann. Appl. Biol.* **56**:73.

Hollings, M., and Stone, O. M., 1970, Carnation ringspot virus, *CMI/AAB Descriptions of Plant Viruses* No. 21.

Hollings, M., and Stone, O. M., 1977, Red clover necrotic mosaic virus, *CMI/AAB Descriptions of Plant Viruses* No. 181.

Hull, R., 1989, The movement of viruses in plants, *Annu. Rev. Phytopathol.* **27**:213.

Inouye, N., and Hiruki, C., 1985, A new strain of sweet clover necrotic mosaic virus isolated from alfalfa, *Ann. Phytopathol. Soc. Japan* **51**:82.

Kalmakoff, J., and Tremaine, J. H., 1967, Some physical and chemical properties of carnation ringspot virus, *Virology* **33**:10.

Kaper, J. M., 1975, *The Chemical Basis of Virus Structure, Dissociation and Reassembly*, North-Holland, Amsterdam.

Kassanis, B., 1955, Some properties of four viruses isolated from carnation plants, *Ann. Appl. Biol.* **43**:103.

Kassanis, B., and Woods, R. D., 1968, Aggregated forms of the satellite of tobacco necrosis virus, *J. Gen. Virol.* **2**:395.

Kassanis, B., White, R. F., and Woods, R. F., 1973, Genetic complementation between middle and bottom components of two strains of radish mosaic virus, *J. Gen. Virol.* **20**:277.

Kegler G., and Kegler, H., 1981, Beiträge zur Kenntnis der vektorlosen Übertragung pflanzenpathogener Viren, *Arch. Phytopathol. Pflanzenschutz* **17**:307.

Kegler, H., Kleinhempel, H., and Stanarius, A., 1984, Evidence of infectious plant viruses after passage through the rodents alimentary tract, *Arch. Phytopathol. Pfanzenschutz* **20**:189.

Kendall, T. L., and Lommel, S. A., 1992, Nucleotide sequence of carnation ringspot dianthovirus RNA-2, *J. Gen. Virol.* **73**:2479.

Kim, K. H., and Lommel, S. A., 1994, Identification and analysis of the site of -1 ribosomal frameshifting in red clover necrotic mosaic virus, *Virology* **200**:574.

Kleinhempel, H., Gruber, G., and Kegler, H., 1980, Investigations on carnation ringspot virus in fruit trees, *Acta Phytopathol. Acad. Sci. Hung.* **15**:107.

Koenig, R., 1986, Plant viruses in rivers and lakes, *Adv. Virus Res.* **31**:321.

Koenig, R., 1988, Detection in surface waters of plant viruses with known and unknown natural hosts, in: *Viruses with Fungal Vectors* (J. I. Cooper and M. J. C. Asher, eds.), pp. 307–313, Association of Applied Biologists, Wellesbourne, UK.

Koenig R., and Lesemann, D.-E., 1985, Plant viruses in German rivers and lakes. 1. Tombusviruses, a potexvirus and carnation mottle virus, *Phytopathol. Z.* **112**:105.

Koenig, R., An, D., Lesemann, D.-E., and Burgermeister, W., 1988, Isolation of carnation ringspot virus from a canal near a sewage plant: cDNA hybridization analysis, serology and cytopathology, *J. Phytopathol.* **121**:346.

Koenig, R., Rüdel, M., and Lesemann, D.-E., 1989, Detection of petunia asteroid mosaic, carnation ringspot and tobacco necrosis viruses in ditches and drainage canals in a grapevine-growing area of West Germany, *J. Phytopathol.* **127**:169.

Koonin, E. V., 1991, The phylogeny of RNA-dependent RNA polymerases of positive-stranded RNA viruses, *J. Gen. Virol.* **72**:2197.

Koonin, E. V., and Dolja, V. V., 1993, Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences, *Crit. Rev. Biochem. Mol. Biol.* **28**:375.

Kowalska, A., 1974, Freeing carnation plants from viruses by meristem-tip culture, *Phytopathol. Z.* **79**:301.

Kühne, T., and Eisbein, K., 1983, Untersuchungen zur Stabilität des Nelkenringflecken-Virus, *Acta Phytopathol. Acad. Sci. Hung.* **18**:101.

Kühne, T., Proll, E., and Reichenbacker, D., 1983, Aggregationseigenschaften des Nelkenringflecken-Virus *in vitro*, *Acta Phytopathol. Acad. Sci. Hung.* **18**:85.

Lane, L. C., 1974, The bromoviruses, *Adv. Virus Res.* **19**:151.

Lange, L., 1977, Interrelations between fungi and viruses, *Plantesygdomme i Denmark 1976* **92**:68.

Lewis, G. C., Heard, A. J., Gutteridge, R. A., Plumb, R. G., and Gibson, R. W., 1985, The effects of mixing Italian ryegrass (*Lolium multiflorum*) with perennial ryegrass (*L. perenne*) or red clover (*Trifolium pratense*) on the incidence of viruses, *Ann. Appl. Biol.* **106**:483.

Lommel, S. A., 1983, *Genome and Replicative Organization of Plant Dianthoviruses and Arkansas Bee Virus*, Ph.D. Thesis, University of California, Berkeley.

Lommel, S. A., McCain, A. H., Mayhew, D. E., and Morris, T. J., 1983, Survey of commercial carnation cultivars for four viruses in California by indirect enzyme-linked immunosorbent assay, *Plant Dis.* **67**:53.

Lommel, S. A., Weston-Fina, M., Xiong, Z., and Lomonossoff, G. P., 1988, The nucleotide sequence and gene organization of red clover necrotic mosaic virus RNA-2, *Nucleic Acids Res.* **16**:8587.

Longworth, J. F., 1978, Small isometric viruses of invertebrates, *Adv. Virus Res.* **23**:103.

Lucas, W. J., and Wolf, S., 1993, Plasmodesmata: The intercellular organelles of green plants, *Trends Cell Biol.* **3**:308.

Lyness, E. W., and Teakle, D. S., 1981, Red clover necrotic mosaic virus isolated from *Trifolium repens* and *Medicago sativa* in Victoria, *Australasian Plant Pathol.* **10**:6.

MacFarlane, I., 1981, Putative vectors of RCNMV, *Rep. Rothamsted Exp. Stn. 1980*:188.

MacFarlane, I., 1982, Virus transmission by fungi: Red clover necrotic mosaic virus (RCNMV), *Rep. Rothamsted Exp. Stn. 1981*:190.

McLean, M. A., Campbell, R. N., Hamilton R. I., and Rochon, D. M., 1994, Involvement of the cucumber necrosis virus coat protein in the specificity of fungus transmission by *Olpidium bornovanus*, *Virology* **204**:840.

Mise, K., Allison, R. F., Janda, M., and Ahlquist, P., 1993, Bromovirus movement protein genes play a crucial role in host specificity, *J. Virol.* **67**:2815.

Mor, Y., 1983, Carnation trends in Israel, *Acta Hortic.* **141**:253.

Morales, F., Castaño, M., Calvert, L., and Arroyave, J. A., 1992, Furcraea necrotic streak virus: An apparent new member of the dianthovirus group, *J. Phytopathol.* **134**:247.

Morris, T. J., 1983, Virus-specific double-stranded RNA: Functional role in RNA virus infection, in: *Plant Infectious Agents: Viruses, Viroids, Virusoids and Satellites* (H. D. Robertson, S. H. Howell, M. Zaitlin, and M. Malmberg, eds.), pp. 80–83, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Morris-Krsinich, B., Forster, R. L., and Mossop, D. W., 1983, Translation of red clover necrotic mosaic virus RNA in rabbit reticulocyte lysate: identification of the virus coat protein cistron on the larger RNA strand of the bipartite genome, *Virology* **124**:349.

Musil, M., 1969a, Red clover necrotic mosaic virus, a new virus infecting red clover (*Trifolium pratense*) in Czechoslovakia, *Biologica (Bratislava)* **24**:33.

Musil, M., 1969b, Serological properties of certain isolates of red clover necrotic mosaic virus, *Acta Virol.* **13**:226.

Musil, M., and Gallo, J., 1982, Serotypes of red clover necrotic mosaic virus. I. Characterization of three serotypes, *Acta Virol.* **26**:497.

Musil, M., and Matisova, J., 1967, A contribution to the knowledge of virus mosaic of red clover in Slovakia, *Ochrana Rostlin N.S.* **3**:225.

Musil, M., Leskova, O., and Smrz, J., 1979, Susceptibility and reaction of some Leguminosae to viruses occurring in Czechoslovakia, *Sbornik Vedeckych Praci.* **5/6**:205.

Musil, M., Leskova, O., and Gallo, J., 1982, Serotypes of red clover necrotic mosaic virus. II. Typing of 34 isolates, *Acta Virol.* **26**:502.

Noordam, D., Thung, T. H., and Van der Want, J. P. H., 1951, Investigations on carnation mosaic. I. *Tidjschr. Pl. Zeikt.* **57**:72.

Okuno, T., Hiruki, C., Rao, D. V., and Figueiredo, G. C., 1983, Genetic determinants distributed in two genomic RNAs of sweet clover necrotic mosaic, red clover necrotic mosaic and clover primary leaf necrosis viruses, *J. Gen. Virol.* **64**:1907.

Orlova, E. V., Tsuprun, V. L., Kiselev, N. A., and Spaar, D., 1980, Structure of carnation ringspot virus and its crystal monolayers, *Electron Microsc.* **2**:602.

Osman, T. A. M., and Buck, K. W., 1987, Replication of red clover necrotic mosaic virus RNA in cowpea protoplasts: RNA 1 replicates independently of RNA 2, *J. Gen. Virol.* **68**:289.

Osman, T. A. M., and Buck, K. W., 1991a, Double-stranded RNAs isolated from plant tissue infected with red clover necrotic mosaic virus correspond to genomic and subgenomic single-stranded RNAs, *J. Gen. Virol.* **71**:945.

Osman, T. A. M., and Buck, K. W., 1991b, Detection of the movement protein of red clover necrotic mosaic virus in a cell wall fraction from infected *Nicotiana clevelandii* plants, *J. Gen. Virol.* **72**:2853.

Osman, T. A. M., Dodds, S. M., and Buck, K. W., 1986, RNA 2 of red clover necrotic mosaic virus determines lesion morphology and systemic invasion of cowpea, *J. Gen. Virol.* **67**:203.

Osman, T. A. M., Miller, S. J., Marriott, A. C., and Buck, K. W., 1991a, Nucleotide sequence of RNA-2 of a Czechoslovakian isolate of red clover necrotic mosaic virus, *J. Gen. Virol.* **72**:213.

Osman, T. A. M., Ingles, P. J., Miller, S. J., and Buck, K. W., 1991b, A spontaneous red clover necrotic mosaic virus mutant with a truncated movement protein, *J. Gen. Virol.* **72**:1793.

Osman, T. A. M., Hayes, R. J., and Buck, K. W., 1992, Cooperative binding of the red clover necrotic mosaic virus movement protein to single-stranded nucleic acids, *J. Gen. Virol.* **73**:223.

Paje-Manalo, L. L., and Lommel, S. A., 1989, Independent replication of red clover necrotic mosaic virus RNA-1 in electroporated host and nonhost *Nicotiana* species protoplasts, *Phytopathology* **79**:457.

Pappu, H. R., and Hiruki, C., 1989, Electrophoretic variability among dianthoviruses, *Phytopathology* **79**:1253.

Pappu, H. R., Hiruki, C., and Inouye, N., 1988, A new serotype of sweet clover necrotic mosaic virus, *Phytopathology* **78**:1343.

Prüfer, D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A., and Rodhe, W., 1992, Ribosomal frameshifting in plants: A novel signal directs the -1 frameshift in the synthesis of the putative viral replicase of potato leafroll virus, *Embo J.* **11**:1111.

Ragetli, H. W. J., and Elder, M., 1977, Characteristics of clover primary leaf necrosis virus, a new spherical isolate from *Trifolium pratense*, *Can. J. Bot.* **55**:2122.

Rao, A. L. N., and Hiruki, C., 1985, Clover primary leaf necrosis virus: A strain of red clover necrotic mosaic virus, *Plant Dis.* **69**:959.

Rao, A. L. N., and Hiruki, C., 1987, Unilateral compatibility of genome segments from two distinct strains of red clover necrotic mosaic virus, *J. Gen. Virol.* **68**:191.

Rao, A. L. N., Ohki, S. T., Lakshman, D. K., and Hiruki, C., 1987, Identification of a new serotype and antigenic relationships among six isolates of red clover necrotic mosaic virus: Identification of a new serotype, *Phytopathology* **77**:995.

Richter, J., Kleinhempel, H., Gruber G., and Kegler, H., 1978, Identifizierung eines Virus von

Birnen mit Steinfruchtigkeit als Nelkenringflecken-Virus (carnation ringspot virus), *Arch. Phytopathol. Pflanzenschutz* **14:**411.
Rochon, D. M., Johnston, J. C., and Rivière, C. J., 1991, Molecular analysis of the cucumber necrosis virus genome, *Can. J. Plant Pathol.* **6:**142.
Rüdel, M., Querfurth, G., and Paul, H. L., 1977, Natürliches Vorkommen des carnation ringspot virus in *Stellaria media* (L.) Cyrill. aus Weinbergen erstmals nachgewiessen, *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes, Stuttgart:* **29:**59.
Ryabov, E. V., Generozov, E. V., Kendall, T. L., Lommel, S. A., and Zavriev, S. K., 1994, Nucleotide sequence of carnation ringspot dianthovirus RNA-1, *J. Gen. Virol.* **75:**243.
Sparnaaij, L. D., 1983, Carnations in Europe, *Acta Hortic.* **141:**261.
Stone, O. M., 1968, The elimination of four viruses from carnation and sweet william by meristem-tip culture, *Ann. Appl. Biol.* **62:**119.
Tomlinson, J. A., and Faithfull, E. M., 1984, Studies on the occurrence of tomato bushy stunt virus in English rivers, *Ann. Appl. Biol.* **104:**485.
Tomlinson, J. A., Faithfull, E. M., Flewett, T. H., and Beards, G., 1982, Isolation of infective tomato bushy stunt virus after passage through the human alimentary tract, *Nature* **300:**637.
Tomlinson, J. A., Faithfull, E. M., and Seeley, N. D., 1983a, Plant viruses in rivers, *Rep. Natl. Vegetable Res. Stn. 1982*:86.
Tomlinson, J. A., Faithfull, E. M., Webb, M. J. W., Fraser, R. S. S., and Seeley, N. D., 1983b, Chenopodium necrosis: A distinctive strain of tobacco necrosis virus isolated from river water, *Ann. Appl. Biol.* **102:**135.
Tosic, M., and Tosic, D., 1984, Occurrence of tobacco mosaic virus in water of the Danube and Sava rivers, *Phytopathol. Z.* **110:**200.
Tremaine, J. H., 1961, Removal of host antigens from plant virus preparations by ion exchange chromatography, *Can. J. Bot.* **39:**1705.
Tremaine, J. H., and Dodds, J. A., 1985, Carnation ringspot virus, *AAB Descriptions of Plant Viruses* No. 308.
Tremaine, J. H., and Ronald, W. P., 1976, Differential effects of sodium dodecyl sulphate on strains of carnation ringspot virus, *J. Gen. Virol.* **30:**299.
Tremaine, J. H., and Ronald, W. P., 1985, The effect of pH and some selected chemicals on the temperature-reversible aggregation of carnation ringspot virus, *Phytopathology* **75:**467.
Tremaine, J. H., Ronald, W. P., and Valcic, A., 1976, Aggregation properties of carnation ringspot virus, *Phytopathology* **66:**34.
Tremaine, J. H., Ronald, W. P., and McGauley, E. M., 1983, Effect of sodium dextran sulfate on some isometric plant viruses, *Phytopathology* **73:**1241.
Tremaine, J. H., Ronald, W. P., and McGauley, E. M., 1984, Temperature-reversible aggregation of two strains of carnation ringspot virus, *Phytopathology* **74:**161.
Valenzuela, M., and Pizano, M., 1992, Distribution of carnation mottle virus and carnation ringspot virus in the Bogota Sabana, *Acta Hortic.* **307:**221.
Van Regenmortel, M. H. V., 1982, *Serology and Immunochemistry of Plant Viruses*, Academic Press, New York.
Van Regenmortel, M. H. V., and Burckard, J., 1980, Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme-linked immunosorbent assays, *Virology* **106:**327.
Varmus, H., 1988, Retroviruses, *Science* **240:**1427.
Veidt, I., Lot, H., Scheidecker, D., Guilley, H., Richards, K., and Jonard, G., 1988, Nucleotide sequence of beet western yellows virus RNA, *Nucleic Acids Res.* **16:**9917.
Weintraub, M., Ragetli, H. W. J., and Leung, E., 1975, Abnormal nuclear structures in mesophyll cells infected with carnation ringspot virus, *Phytomorphology* **25:**288.
Xiong, Z., and Lommel, S. A., 1989, The complete nucleotide sequence and genome organization of red clover necrotic mosaic virus RNA-1, *Virology* **171:**543.
Xiong, Z., and Lommel, S. A., 1991, Red clover necrotic mosaic virus infectious transcripts synthesized *in vitro*, *Virology* **182:**388.

Xiong, Z., Kim, K. H., Giesman-Cookmeyer, D., and Lommel, S. A., 1993a, The roles of the RCNMV capsid and cell-to-cell movement proteins in systemic infection, *Virology* **193**:27.

Xiong, Z., Kim, K. H., Kendall, T. L., and Lommel, S. A., 1993b, Synthesis of the putative red clover necrotic mosaic virus RNA polymerase by ribosomal frameshifting *in vitro*, *Virology* **193**:213.

Yi, L., Lesemann, D.-E., Koenig, R., Rüdel, M., and Pfeilstetter, E., 1992, Isometric plant viruses found in ditches and streams in agricultural areas: recovery of previously found viruses and identification of hitherto unrecorded carmo- and tombusviruses including grapevine Algerian latent virus, *J. Phytopathol.* **134**:121.