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Nicotiana velutina mosaic virus: evidence for a bipartite genome comprising 3 kb and 8 kb RNAs

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DNA complementary to *Nicotiana velutina* mosaic virus (NVMV) RNA was cloned and five segments larger than 0·9 kb were used in Northern blot hybridization analysis to identify two virus-specific RNAs, approximately 8 kb (RNA 1) and 3 kb (RNA 2) in size. The clones selected as probes did not hybridize with RNA from various tobamoviruses, or from beet necrotic yellow vein (BNYVV) and peanut clump furoviruses. In an attempt to determine the taxonomic position of the virus, about 75% of the NVMV RNA 2 was sequenced and four open reading frames (ORFs) were identified. ORFs 1, 2 and 3 encode proteins of M_r 20K, 39K and 13K, whereas ORF 4 was incomplete. ORFs 2, 3 and 4 overlapped in an arrangement closely resembling the triple gene block identified in BNYVV RNA 2, barley stripe mosaic virus (BSMV) RNA 2, potato virus X and potato virus M RNA. The presumed coat protein gene of NVMV RNA 2 (ORF 1) is situated to the 5′ side of the triple gene block as for BNYVV and BSMV RNA 2. Amino acid homologies were detected among the 13K and 14K proteins of NVMV RNA 2, BNYVV RNA 2 and BSMV RNA 2. Significant homology was also detected between the 39K protein of NVMV RNA 2 and the 42K protein of BNYVV RNA 2, with a motif specific for ATP- and GTP-binding (NTP-binding motif), and a conserved viral DNA polymerase domain. The presence of a triple gene block in NVMV RNA 2 indicates that NVMV has affinities with members of the hordei-, furo-, potex- and carlavirus groups but not with the tobamovirus group. The divided RNA genome of NVMV, and the sizes of the two RNAs suggest that NVMV is most closely allied to the furoviruses, but the unique nature of its different biological properties and lack of any serological relationships with furoviruses lead us to conclude that NVMV has no clear relatedness to any taxonomic group of plant viruses.

Introduction

Nicotiana velutina mosaic virus (NVMV) was isolated from a single *N. velutina* plant with mosaic symptoms in the semi-arid zone of South Australia (Randles *et al.*, 1976; Randles, 1978). It is mechanically transmissible to a narrow host range. No vector is known but a high rate of seed transmission in several *Nicotiana* spp. may account for its survival in the field. The rigid rod-shaped particles fragment during purification, and although particles of up to 700 nm long have been seen, the modal length of partially purified particles is 125 to 150 nm. There is also a wide range of other particle sizes.

The taxonomic position of NVMV is unknown. Particle morphology (a helical capsid of 2·9 nm pitch, diameter of 18 nm) and coat protein M_r value 21400 (Randles *et al.*, 1976) could place NVMV in either the tobamo-, hordei-, tobravirus (Matthews, 1982), or furovirus (fungus-transmitted labile rod-shaped viruses with a bipartite genome; Brunt & Shikata, 1986; Brunt & Richards, 1989; Brown, 1989) groups. However, no serological relationship to tobacco mosaic tobamovirus (TMV), beet necrotic yellow vein (BNYVV), soil-borne wheat mosaic (SBWMV), or potato mop top furoviruses, barley stripe mosaic hordeivirus (BSMV) or tobacco rattle tobravirus has been detected (Randles *et al.*, 1976).

The number of segments into which the genome is divided and their size may assist in placing rod-shaped viruses in a tentative taxonomic group (Dougherty & Hiebert, 1985). Tobamoviruses have one genomic RNA segment, furoviruses two to four, hordeiviruses three and tobraviruses two. The longest RNA component occurs in the furoviruses (6·7 kb; Bouzoubaa *et al.*, 1987) and tobamoviruses (6·4 kb; Dougherty & Hiebert, 1985). Early attempts to analyse the RNA content of NVMV showed a component with an M_r of approx. $2{\cdot}3 \times 10^6$ (approx. 6·5 kb) with up to eight smaller RNA species (Randles, 1978). Such a complex pattern is unknown for viruses, and was of no value for assigning NVMV to any known taxonomic group. It is unlikely that each RNA

Table 1. *Viruses compared with NVMV by nucleic acid hybridization*

Virus preparation	Source
Tobacco mosaic virus U1	Waite Institute Collection
U2	
U5	
U6	
U8	
U5-TMV from *N. glauca*, 12 isolates	
Frangipani mosaic virus	
Odontoglossum ringspot virus	
Tomato mosaic virus (Dahlemense)	
Sunnhemp mosaic virus	
Cucumber green mottle mosaic virus 4	
Kyuri green mottle mosaic virus (Francki *et al.*, 1986)	
TMV petunia isolate	
Arachis hypogea leaf infected with peanut clump virus strain K84	M. Dollet, CIRAD, Montpellier, France
strain N'Bour/2	
Chenopodium quinoa leaf infected with beet necrotic yellow vein virus 89/1	R. Koenig, BBA, Braunschweig, F.R.G.
N. glauca leaf infected with U5-TMV	Field collection, Almeria, Spain

represents a unique genome segment, and we therefore approached the problem of defining the genome organization of NVMV by cloning NVMV RNA and using selected non-overlapping clones in Northern blot analysis to identify unique genome components of the virus.

Methods

NVMV preparation. The method of Randles *et al.* (1976) was modified as follows. Systemically infected *N. benthamiana* leaves (maintained at 25 °C under constant light) were triturated with a pestle and mortar in three volumes (w/v) of 67 mM-phosphate buffer pH 5·6, containing 10 mM-sodium diethyldithiocarbamate and 0·1% thioglycerol. The mixture was strained through muslin and centrifuged at 10000 *g* for 10 min. The pellet, which contains the virus, was resuspended in 50 mM-glycine buffer pH 9·0, containing 0·05% thioglycerol (GT buffer) by agitation for 150 min at 4 °C, then clarified by centrifugation at 10000 *g* for 10 min. Virus in the supernatant fraction was centrifuged through a 40% sucrose cushion containing GT buffer, at 50000 r.p.m. for 90 min in the 70 Ti Beckman rotor. The pellet was resuspended in 0·2 × GT buffer, and clarified by shaking with 1 volume of chloroform for 10 min, and low-speed centrifugation. Virus in the upper phase was sedimented at 70000 r.p.m. for 20 min in the TLA 100.3 Beckman rotor, and the pellet was resuspended in 0·2 × GT buffer.

The resuspended preparation was layered on a 3 ml 30 to 60% gradient of Nycodenz (Nyegaard) dissolved in 10 mM-Tris–HCl pH 7·2, and subjected to isopycnic density gradient centrifugation for 16 h at 50000 r.p.m. at 5 °C in the TLA 100.3 Beckman rotor. The single light-scattering band three-fifths of the way down the gradient was removed, diluted in 0·2 × GT and virus was again pelleted by high-speed centrifugation. Virus was resuspended in buffer and lyophilized for storage.

Nucleic acid preparations

(i) *Virus RNA.* RNA was prepared from NVMV and the viruses listed in Table 1 by incubating preparations in 10 mM-Tris–HCl pH 7·5, 0·5% SDS, containing 200 mM-sodium acetate and proteinase K (50 μg/ml), for at least 3 h at 37 °C. Mixtures were extracted once with an equal volume of water-saturated phenol, and RNA was precipitated with ethanol. Peanut leaf infected with two isolates of peanut clump virus, *Chenopodium quinoa* leaf infected with BNYVV, and *N. glauca* leaf naturally infected with U5-TMV, were ground with 4 volumes (w/v) each of 200 mM-Tris–HCl pH 7·5 containing 2% SDS and phenol. The extract was shaken for 1 h, centrifuged, and the supernatant fraction was re-extracted with phenol, then chloroform, and RNA was precipitated with ethanol.

(ii) *NVMV RNA from the pH 5·6 low-speed centrifugation pellet.* Nucleic acids were extracted from the NVMV-enriched low-speed pellet following blending in pH 5·6 buffer (see above). This pH 5·6 pellet was resuspended in 100 mM-Tris–HCl pH 8·0, containing 1% SDS, and mixed with an equal volume of water-saturated phenol. After shaking for 60 min, and low-speed centrifugation, the supernatant fraction was re-extracted with phenol, then twice with chloroform. Nucleic acids were precipitated with ethanol.

(iii) *Total nucleic acids.* Systemically infected leaves were blended in a polytron mixer with 3 to 5 volumes each of phenol, and a buffer containing 50 mM-MOPS pH 7·0, 50 mM-EDTA, 2 M-urea, 1% Triton X-100 and 2% 2-mercaptoethanol. The mixture was shaken for 15 min, centrifuged at 5000 *g* for 10 min and the aqueous supernatant fraction was extracted once with phenol, and once with chloroform. Nucleic acids were precipitated with 2·5 volumes of ethanol in the presence of 0·3 M-sodium acetate.

Qiagen fractionation of total nucleic acids. To remove polysaccharides and DNA from total nucleic acids, preparations were mixed with NaCl to 350 mM, then passed through a Qiagen pack 20 column equilibrated with buffer containing 400 mM-NaCl, 50 mM-MOPS pH 7·0 and 15% ethanol (Diagen). The absorbed nucleic acids were washed with the equilibration buffer, and the RNA fraction was then eluted with the same buffer modified by adding NaCl to 1·1 M and urea to 2 M. RNA was precipitated with 0·8 volumes of isopropanol. Nucleic acid precipitates were washed with ethanol, dried, and dissolved in 10 mM-Tris–HCl pH 7·5, 0·1 mM-EDTA (TE).

Cloning of NVMV RNA. Cloning was done essentially as described by Schwarz-Sommer *et al.* (1985). cDNA was reverse-transcribed from NVMV RNA with Moloney murine leukaemia virus (MMuLV) reverse transcriptase [Gibco-Bethesda Research Laboratories (BRL)],

second strand synthesis was done with DNA polymerase I in the presence of DNA ligase and RNase H, and single-stranded ends were removed with mung bean nuclease (Pharmacia). *Eco*RI sites were methylated with *Eco*RI methylase, repair was done with Klenow polymerase, which was followed by a second mung bean nuclease digestion. Phosphorylated *Eco*RI linkers were added in the presence of T4 DNA and RNA ligases, the DNA was treated with *Eco*RI restriction endonuclease and high M_r DNA was separated by chromatography on Sepharose 4B. The DNA was ligated into *Eco*RI-cut phage λ NM1149 DNA with T4 DNA ligase, and the λ DNA was packaged and inoculated to POP 13 *Escherichia coli* cells. About 600000 recombinant phages were obtained. After plating and incubation, plaques were lifted and probed with cDNA to NVMV RNA. Two cycles of selection were done and 40 single plaques were isolated at random after the second selection. Eleven of those with large inserts were amplified by infecting POP 13 cells. λ DNA was prepared from each and *Eco*RI digests were analysed by Southern blot analysis. Inserts were isolated by agarose gel electrophoresis, electroelution, then bound to DEAE-cellulose (DE52, Whatman) columns in the presence of TE buffer, and finally eluted with 1 M-NaCl in TE buffer. Following precipitation with ethanol, each DNA was tested for homology against cDNAs prepared to all the others using dot blot hybridization analysis.

The fragments were subcloned by ligation into *Eco*RI-cut pSP65 plasmids which were then introduced into competent MC1061 *E. coli* cells. Transformed colonies were identified with cDNA specific for NVMV RNA and a second cycle of plating was done. Selected colonies were grown up to produce mini-preparations of plasmid.

cDNA synthesis. To synthesize cDNA probes for hybridization assay, cloned inserts were excised from the plasmid with *Eco*RI, then isolated by agarose gel electrophoresis, electroelution, and DE52 chromatography as described above. For radiolabelling (Feinberg & Vogelstein, 1984) approximately 500 ng of DNA in water was denatured at 100 °C for 15 min, then incubated in the presence of hexa-deoxynucleotide primers (250 μg/ml; Pharmacia), 10 mM-$MgCl_2$, 50 mM-Tris-HCl pH 7·5, 50 μM-dATP, -dGTP and -dTTP, 50 μCi[^{32}P]dCTP (3000 Ci/mmol), 7 mM-2-mercaptoethanol, and either MMuLV reverse transcriptase (400 units) or Klenow polymerase (2 units). The reaction mixture volume was 50 μl, with incubation for 5 h at either 37 °C (reverse transcriptase) or 25 °C (Klenow). The transcribed probe was separated on G50 Sephadex columns, boiled in TE buffer, and added to hybridization buffer to give between $0{\cdot}1 \times 10^6$ and $1{\cdot}5 \times 10^6$ c.p.m./ml.

Hybridization assays. For dot blot analysis, nucleic acid extracts dissolved in TE buffer were applied as 1 μl spots to 0·2 μm pore size nitrocellulose membranes (Schleicher & Schuell; BA83-20) which had been previously washed in water and 20 × SSC, then air-dried. Membranes were baked at 80 °C for 2 h, prehybridized at 68 °C for at least 1 h in 6 × SSPE (SSPE is 180 mM-NaCl, 10 mM-sodium phosphate, 1 mM-EDTA pH 7) containing 0·02% Ficoll (M_r 40000), 0·02% polyvinylpyrrolidone (M_r 350000), 0·1% SDS and boiled salmon sperm DNA (50 μg/ml). Hybridization buffer was as above but contained 3 × SSPE, 20 μg/ml boiled carrier DNA and boiled cDNA. Incubation was at 68 °C for at least 16 h. Membranes were washed for at least 1 h in 2 × SSPE, 0·1% SDS at 68 °C before autoradiography. Higher stringency washes were done in either 1 × SSPE or 0·1 × SSPE at 68 °C.

RNA for Northern blot analysis was incubated at 65 °C for 5 min in 50% formamide and 6% formaldehyde, which was buffered with 20 mM-MOPS containing 5 mM-sodium acetate and 1 mM-EDTA pH 7·0. Electrophoresis was in 1·2% agarose gels containing 6·3% formaldehyde, and the same MOPS buffer. A 0·3 to 9·5 kb RNA ladder (BRL) was coelectrophoresed as a size marker, using ethidium bromide or toluidine blue to stain the bands. RNA was transferred to a nylon membrane (Hybond N; Amersham) by capillary blotting in 20 × SSPE for at least 16 h, fixed by u.v. irradiation for 5 min and baking at 80 °C for 2 h. It was subjected to the prehybridization, hybridization and washing steps described above. After autoradiography, cDNA was removed by boiling in 0·1% SDS for 30 min before repeating the prehybridization and hybridization steps with another cDNA. Probe solutions in hybridization buffer were boiled before re-use.

Sequencing. *Eco*RI restriction fragments 10A, 10B and 10C generated from λ clone NL10 DNA were subcloned into the *Eco*RI site of pSP65. Sequences of appropriate restriction fragments were determined for both orientations according to Maxam & Gilbert (1980). Sequences across the internal *Eco*RI sites of NL10 were obtained by sequencing *Sty*I fragments released from NL10 DNA and purified by agarose gel electrophoresis.

Results

Gel electrophoretic analysis of NVMV RNA

Preparations of NVMV RNA showed numerous bands on silver-stained polyacrylamide gels. Some bands were more heavily stained than others and a comparison of patterns from different preparations showed that some bands were common for these preparations, whereas others were variable in occurrence. In denaturing gels containing 8 M-urea, and using single-stranded DNA M_r markers, the smaller RNA bands ranged in size from 36 to over 800 nucleotides. Such variation in pattern and the large number of bands may be due to the fragmentation of NVMV particles into smaller particles during purification (Randles *et al.*, 1976) and the subsequent isolation of RNAs of varying sizes from this range of particle fragments.

The nature of the genome of NVMV was therefore investigated by preparing a cDNA library of RNA isolated from purified virus, and by identifying specific inserts for use in detecting NVMV RNAs in Northern blot analyses of virus-infected plants.

Cloning of NVMV RNA

The clones isolated from phage λNM1149 by *Eco*RI digestion ranged in size from 0·9 to 3·2 kb. The pattern of cross-hybridization (Table 2) showed that the inserts could be placed in separate groups without detectable cross-homology. The following inserts were selected for the synthesis of probes: 8 (in λNM1149), 10A, 10B, 14C and 16A (subcloned in pSP65).

Northern blot analysis

Northern blot analysis was done with RNA isolated from purified NVMV, the pH 5·6 pellet of infected plant sap (which is enriched with NVMV but subjected to minimum manipulation) and total nucleic acids extracted by either phenol-SDS extraction, or the Qiagen

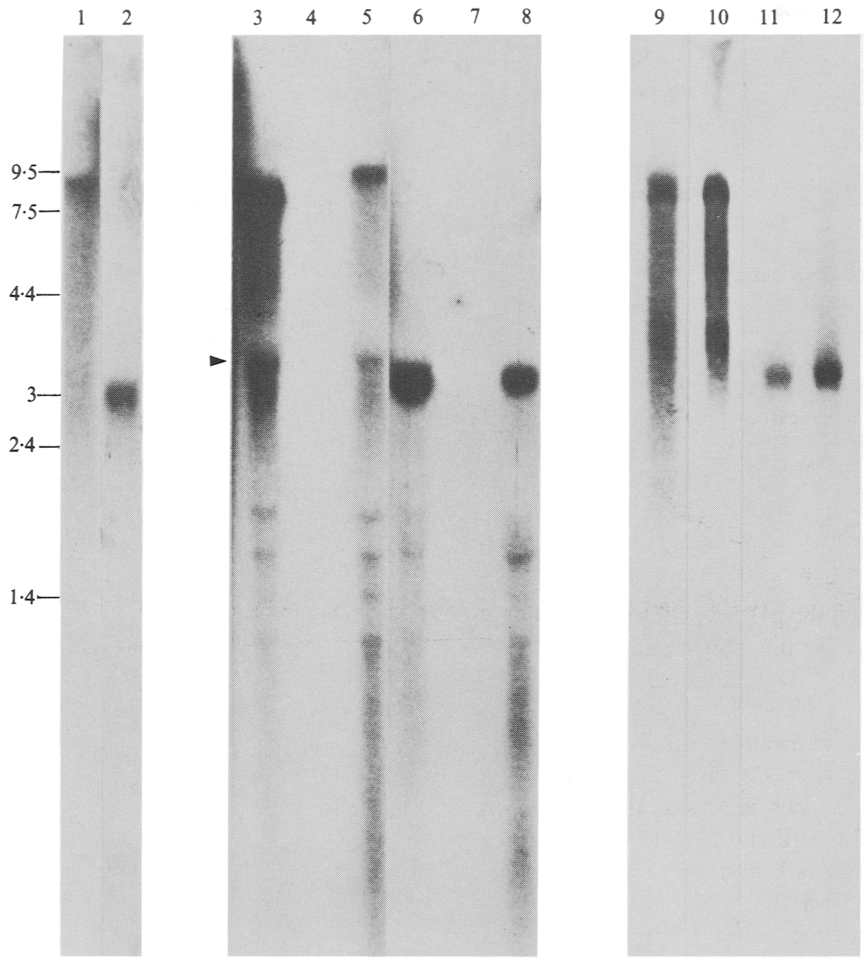


Fig. 1. Northern blot analysis of RNA isolated from purified NVMV (lanes 1 and 2), systemically infected (lanes 3, 5, 6 and 8) or healthy leaf (lanes 4 and 7) and the pH 5·6 pellet from infected *N. glutinosa* (lanes 9 and 11) and *N. benthamiana* leaf (lanes 10 and 12). Samples in lanes 4, 5, 7 and 8 were subjected to Qiagen fractionation. The 8 kb specific probe was used for lanes 1, 3, 4, 5, 9, 10; 3 kb specific probes were used for lanes 2, 6 7, 8, 11 and 12. An arrowhead marks the 3·1 kb band with homology to the 8 kb RNA. Sizes on the left are in kb.

Table 2. *Inserts specific for NVMV RNA cloned in λNM1149 with their pattern of cross-hybridization as determined by dot blot hybridization*

	cDNA probe											
Insert	7	8	10A	10B	11	12	14B	14C	15	16A	16B	17
7 (2·0)*	+†	+										
8 (3·2)	+	+										
10A (1·1)			+		+	+	+		+		+	+
10B (0·9)				+				+				
11 (2·2)			+		+	+	+		+		+	+
12 (1·1)			+	+	+	+	+		+		+	+
14B (1·2)			+		+	+	+		+		+	+
14C (0·9)				+				+				
15 (1·1)			+	+	+	+	+		+		+	+
16A (1·4)										+		
16B (0·9)			+	+	+	+	+		+		+	+
17 (1·1)			+	+	+	+	+		+		+	+

* Size of insert in kb is shown in parentheses.
† The plus symbol (+) indicates positive hybridization.

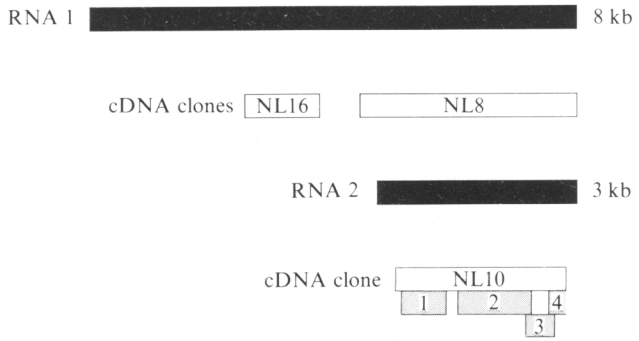


Fig. 2. The putative genome arrangement of NVMV. Sizes of cDNA clones representing either of the two genomic RNAs are indicated but their positioning with respect to the RNAs is tentative. Numbers 1 to 4 within cDNA clone NL10 denote ORFs representing M_r values of 20K (ORF 1), 39K (ORF 2), 13K (ORF 3) and an incomplete sequence representing over 9K (ORF 4).

method. Nucleic acids in the pH 5·6 pellets of infected *N. benthamiana* (mechanically inoculated) and *N. glutinosa* (infected through the seed) were also compared. Appropriate healthy control tissue was included.

RNA from all sources showed two main virus-specific components, 3 kb and 8 kb in size (Fig. 1). No homology was detected between the two RNAs as the 3 kb RNA hybridized to inserts 10A, 10B and 14C, whereas the 8 kb RNA hybridized to inserts 8 and 16A. Minor smaller bands were detected below the major bands with each probe in the total nucleic acids of infected plants. Washing and rehybridizing the blots with a probe representing the reciprocal group showed that the minor band detected in the 3·1 kb position with probe 16A is slightly larger than the 3 kb RNA detected with 10A, 10B or 14C. Thus, the 3·1 kb band probably represents a fragment of the 8 kb RNA. The other minor RNAs detected by probes 10A, 10B and 14C were approximately 2·4, 1·9, 1·6, 1·4, 1·1 and 0·9 kb, whereas those detected by probe 16A were 1·9, 1·6, 1·4 and 1·1 kb.

Tests of homology with other rod-shaped viruses

Dot-blot hybridizations using probes 10A, 10B and 16A showed that they had no detectable homology with the tobamoviruses or furoviruses listed in Table 1 (data not shown). Similarities among rod-shaped plant viruses that are not related serologically or by molecular hybridization may become evident, however, when genome organization and gene sequences are compared (Morozov *et al.*, 1989). We therefore determined the nucleotide sequence for the largest cDNA clone in phage λ, NL10, which represents most of the NVMV RNA 2 (Fig. 2). The three cDNA fragments (10A, 10B and 10C) generated from NL10 by *Eco*RI restriction were sequenced individually and their order was obtained by restriction analysis of NL10 in conjunction with specific probes. Sequences across the internal *Eco*RI sites were established using *Sty*I fragments released from NL10.

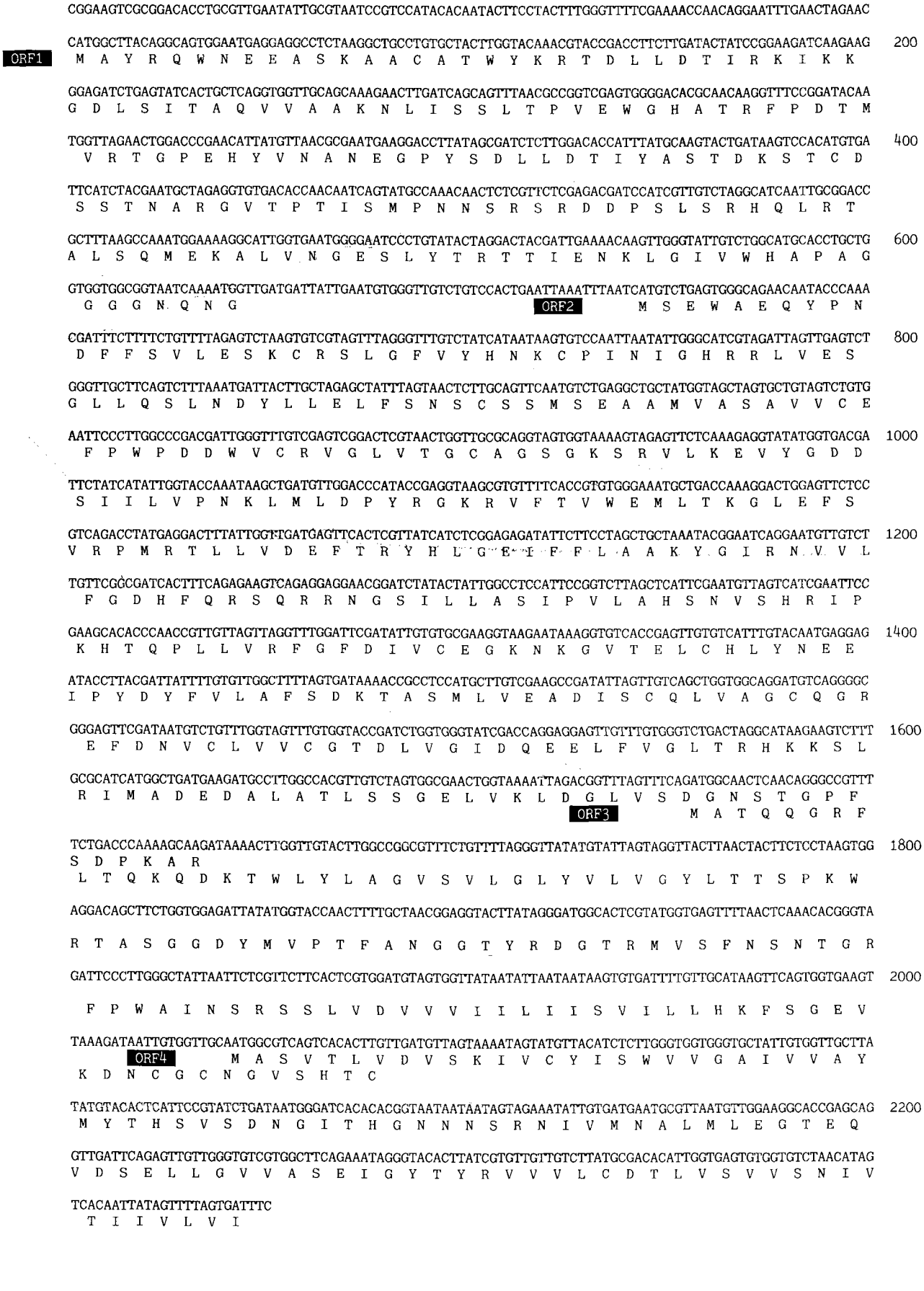
The total sequence of 2·3 kb (Fig. 3) corresponds to approximately 75% of NVMV RNA 2. It contains four open reading frames (ORFs) encoding putative viral proteins larger than 10K of which ORF 4 was not fully represented by NL10 (see also Fig. 2).

There is an overlap between ORF 2 and ORF 3, and between ORF 3 and ORF 4 (Fig. 2 and 3). This arrangement of genes is reminiscent of the gene organization for BNYVV (RNA 2), BSMV (RNA 2), potato virus X (PVX; Morozov *et al.*, 1989) and potato virus M (PVM; Rupasov *et al.*, 1989) (Fig. 6). Moreover, a comparison of the amino acid sequences for the corresponding gene products reveals regions of striking similarity. Fig. 4 shows the alignment of NVMV ORF 2 (39K) with the BNYVV 42K protein. Blocks of conserved amino acids are scattered throughout the whole sequence; some of these (as denoted by asterisks in Fig. 4) are motifs specific for ATP/GTP-binding proteins (NTP motif V.G.AG.GKS; Walker *et al.*, 1982) and viral DNA polymerases (GD at position 214 of BNYVV sequence; Hodgman, 1986) as discussed by Hodgman (1988). This indicates that the NVMV 39K protein might participate in virus replication.

Similarity of the NVMV 13K ORF 3 protein to the BNYVV 13K and BSMV 14K proteins is higher in the N-proximal half of the putative proteins with a central conserved sequence between positions 40 and 70 of the NVMV 13K protein (Fig. 5). This highly conserved sequence is flanked by blocks of hydrophobic residues which suggest an association with membranes in infected cells as noted before for the corresponding proteins of BNYVV, BSMV and PVX (Morozov *et al.*, 1987, 1989). Similarities are also observed between NVMV ORF 4 and the BNYVV 15K, BSMV 17K and PVX 8K proteins, respectively (data not shown).

Discussion

Two non-homologous RNAs approximately 3 kb and 8 kb in size have been identified in purified NVMV (Fig. 1, lanes 1 and 2) using three cloned probes specific for the 3 kb RNA, and two specific for the 8 kb RNA. The pH 5·6 pellet from extracted leaf which was enriched with NVMV, but subjected to minimal processing, showed the 3 and 8 kb RNAs, together with a 3·1 kb RNA homologous to 8 kb RNA. Additional minor bands were detected in the total RNA extracts of infected but not healthy leaf, and they may represent either unencapsidated subgenomic RNAs, or deleted forms of RNA


ORF1
ORF2
ORF3
ORF4

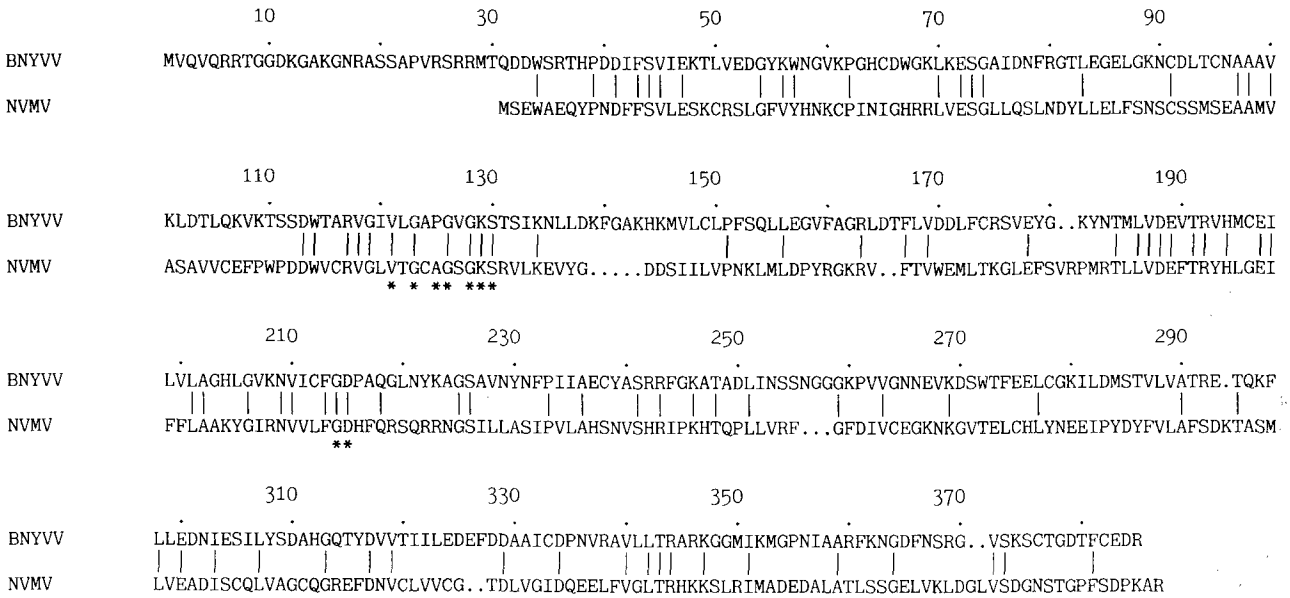


Fig. 4. Alignment of the NVMV 39K product of ORF 2 with the BNYVV 42K protein. Numbering refers to the BNYVV sequence. Identical amino acids are indicated by vertical bars. Asterisks denote motifs of NTP binding and viral DNA polymerase domains as discussed in the text.

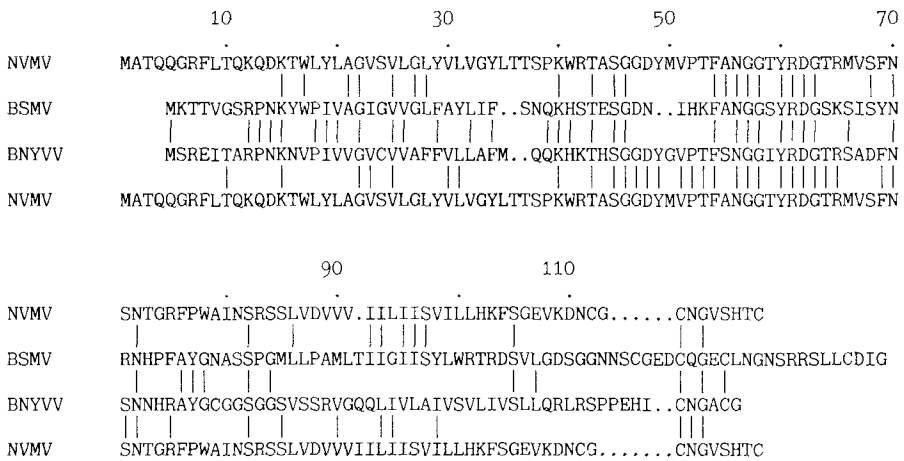


Fig. 5. Sequence comparison of the NVMV 13K putative protein specified by ORF 3 with the BSMV 14K and BNYVV 13K proteins. Numbering refers to the NVMV sequence. Identical amino acids are indicated by vertical bars. Hydrophobic regions within the NVMV sequence are shaded.

similar to those described for SBWMV RNA 2 (Brunt & Richards, 1989).

The mechanically inoculated *N. benthamiana* and seed-infected *N. glutinosa* (Fig. 1, lanes 9 to 12) showed no differences which could be attributable to mode of transmission or to the different host species. The furovirus BNYVV shows variation in the 1·7 kb RNA 3 and 1·4 kb RNA 4 following maintenance in the local lesion host, *Chenopodium quinoa*, but not in RNAs 1 and 2 (Burgermeister *et al.*, 1986).

The lengths of particles encapsidating the 3 and 8 kb RNAs can be estimated if it is assumed that there are 49 nucleotides per turn of the RNA helix (as for TMV and BNYVV; Steven *et al.*, 1981). With a pitch of 2·9 nm (Randles *et al.*, 1976) the particles containing the 3 kb RNA would be 177 nm long, whereas those with the 8 kb

Fig. 3. Nucleotide sequence of the RNA 2-specific cDNA clone NL10. Amino acid sequences for ORF 1 to ORF 4 are given by the one-letter code. Shaded areas indicate internal *Eco*RI sites.

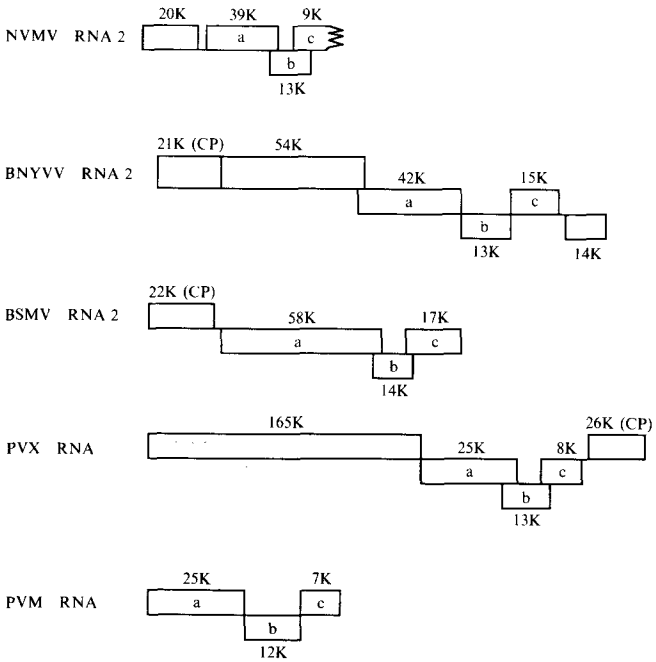


Fig. 6. Organization of NVMV RNA 2 compared with that of BNYVV RNA 2, BSMV RNA 2, the 3′ terminus of PVX RNA (Morozov *et al.*, 1989) and PVM RNA (Rupasov *et al.*, 1989). Protein sizes are shown with similarities annotated as a, b or c.

RNA would be 473 nm. Particle length distributions for NVMV preparations (Randles *et al.*, 1976) showed a minor class with a particle length of about 470 nm and a major class of about 170 nm, which would correspond to the 8 and 3 kb RNAs, respectively. Most particles fell in the range 50 to 180 nm, and as this class increased in relative proportion with increasing purification it seems likely that these particles result from the fragmentation of larger particles.

NVMV is not included with the furoviruses (Brown, 1989) but has been included with the fungus-transmitted and similar labile rod-shaped viruses by Brunt & Shikata (1986). These viruses are serologically distinct from each other. They are superficially similar to authentic tobamoviruses, but they differ in their relative instability *in vitro*, their low concentration in infected plants, and the transmission of most by plasmodiophoromycete fungi. The members which have been characterized have a bipartite genome (Koenig & Huth, 1988; Batista *et al.*, 1989; Brunt & Richards, 1989). Additional RNAs have been identified in BNYVV in which although RNA 1 and RNA 2 are essential for infectivity, RNAs 3 and 4 seem to be required for an ability to infect sugar beet roots systemically (Lemaire *et al.*, 1988; Koenig & Burgermeister, 1989). Therefore the identification in NVMV of 3 and 8 kb RNAs of approximately the same size as described for the genomic RNAs of BNYVV and barley yellow mosaic virus indicates that NVMV may have affinities with the furoviruses and similar labile rod-shaped viruses. No evidence so far links NVMV with any known tobamoviruses. The requirement of the 3 and 8 kb NVMV RNAs for infectivity has not been tested but the identification of these two RNAs in infected plant nucleic acid extracts suggests that these infectivity assays may now be feasible.

Analysis of the sequence of about 75% of NVMV RNA 2 has permitted a more detailed comparison of NVMV with other representatives of groups with rod-shaped particles. Thus, the overlapping triple gene block of NVMV RNA 2 is similar to that on the RNA 2 of BNYVV and BSMV, at the 3′ end of PVX RNA and in PVM RNA (Fig. 6). Moreover, the detectable homologies between the 25K to 58K products, 12K to 14K products and 3′-terminal 7K to 17K products of all five viruses (Fig. 6) indicate that there is marked conservation of genes between viruses of this type. Thus, by analogy, the presence of hydrophobic segments on the putative 13K polypeptide indicate a possible association with membranes (Morozov *et al.*, 1987). The NTP-binding motif in the NVMV 39K polypeptide may have a role in unwinding double-stranded replicative forms during genomic RNA replication (Morozov *et al.*, 1989).

The location of the coat protein gene on the RNA 2 may be a feature of furoviruses (Brunt & Richards, 1989) and if the 20K gene product of NVMV RNA 2 (ORF 1) represents the 21K coat protein of NVMV its position at the 5′ side of the block could indicate similarity with BNYVV, BSMV (Fig. 6) and possibly SBWMV (Brunt & Richards, 1989), but not with PVX.

Sequence similarities may be of limited value in classification because related genomic elements can be found in diverse groups of plant viruses. For example, Morozov *et al.* (1989) have presented evidence that the three genomic RNAs of BSMV each show homology to specific genes of viruses belonging to different virus groups. It is therefore conceivable that NVMV RNA 1 might display phylogenetic relationships to viruses other than those established from an examination of NVMV RNA 2.

In conclusion, therefore, cloning has helped characterize some of the genome organization of NVMV and indicates its possible taxonomic position. NVMV probably has a bipartite genome similar to that of the furoviruses. Identification of the triple gene block allies NVMV with the hordei-, furo-, potex- and carlaviruses, and demonstrates a clear lack of affinity with the tobamoviruses. NVMV seems to be more closely allied to the furoviruses than any other taxonomic group. However, its unique biological properties, a lack of serological relationship to selected furoviruses, and the absence of a known fungal vector, preclude its inclusion in the furovirus group.

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References

BATISTA, M. F., ANTONIW, J. F., SWABY, A. G., JONES, P. & ADAMS, M. J. (1989). RNA/cDNA hybridization studies of UK isolates of barley yellow mosaic virus. *Plant Pathology* **38**, 226–229.

BOUZOUBAA, S., QUILLET, L., GUILLEY, H., JONARD, G. & RICHARDS, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *Journal of General Virology* **68**, 615–626.

BROWN, F. (1989). The classification and nomenclature of viruses: summary of results of meetings of the International Committee on Taxonomy of Viruses in Edmonton, Canada, 1987. *Intervirology* **30**, 181–186.

BRUNT, A. A. & RICHARDS, K. E. (1989). Biology and molecular biology of furoviruses. *Advances in Virus Research* **36**, 1–32.

BRUNT, A. A. & SHIKATA, E. (1986). Fungus-transmitted and similar labile rod-shaped viruses. In *The Plant Viruses*, vol. 2, pp. 305–335. Edited by M. H. V. van Regenmortel & H. Fraenkel-Conrat. New York: Plenum Press.

BURGERMEISTER, W., KOENIG, R., WEICH, H., SEBALD, W. & LESEMANN, D.-E. (1986). Diversity of the RNAs in thirteen isolates of beet necrotic yellow vein virus in *Chenopodium quinoa* detected by means of cloned cDNAs. *Journal of Phytopathology* **115**, 229–242.

DOUGHERTY, W. G. & HIEBERT, E. (1985). Genome structure and gene expression of plant RNA viruses. In *Molecular Plant Virology*, vol. 2, pp. 23–81. Edited by J. W. Davies. Boca Raton: CRC Press.

FEINBERG, A. P. & VOGELSTEIN, B. (1984). A technique for labeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **137**, 266–267.

FRANCKI, R. I. B., HU, J. & PALUKAITIS, P. (1986). Taxonomy of cucurbit infecting tobamoviruses as determined by serological and molecular hybridization analysis. *Intervirology* **26**, 156–163.

HODGMAN, T. C. (1986). An amino acid sequence motif linking viral DNA polymerases and plant virus proteins involved in RNA replication. *Nucleic Acids Research* **14**, 6769.

HODGMAN, T. C. (1988). A new superfamily of replicative proteins. *Nature, London* **333**, 22–23.

KOENIG, R. & BURGERMEISTER, W. (1989). Mechanical inoculation of sugarbeet roots with isolates of beet necrotic yellow vein virus having different RNA compositions. *Journal of Phytopathology* **124**, 249–255.

KOENIG, R. & HUTH, W. (1988). RNA/cDNA hybridization and infectivity tests suggest that barley yellow mosaic virus isolate M has a bipartite genome. *Journal of Phytopathology* **121**, 379–382.

LEMAIRE, O., MERDINOGLU, D., VALENTIN, P., PUTZ, C., ZIEGLER-GRAFF, V., GUILLEY, H., JONARD, G. & RICHARDS, K. (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by *Polymyxa betae*. *Virology* **162**, 232–235.

MATTHEWS, R. E. F. (1982). Classification and nomenclature of viruses. *Intervirology* **17**, 1–199.

MAXAM, A. M. & GILBERT, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods in Enzymology* **65**, 499–560.

MOROZOV, S. YU., DOLJA, V. V. & ATABEKOV, J. G. (1989). Probable reassortment of genomic elements among elongated RNA-containing plant viruses. *Journal of Molecular Evolution* **29**, 52–62.

MOROZOV, S. YU., LUKASHEVA, L. I., CHERNOV, B. K., SKRYABIN, K. G. & ATABEKOV, J. G. (1987). Nucleotide sequence of the open reading frames adjacent to the coat protein cistron in potato virus X genome. *FEBS Letters* **213**, 438–442.

RANDLES, J. W. (1978). *Nicotiana velutina* mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 189.

RANDLES, J. W., HARRISON, B. D. & ROBERTS, I. M. (1976). *Nicotiana velutina* mosaic virus: purification, properties and affinities with other rod-shaped viruses. *Annals of Applied Biology* **84**, 193–204.

RUPASOV, V. V., MOROZOV, S. YU., KANYUKA, K. V. & ZAVRIEV, S. K. (1989). Partial nucleotide sequence of potato virus M RNA shows similarities to potexviruses in gene arrangement and the encoded amino acid sequences. *Journal of General Virology* **70**, 1861–1869.

SCHWARZ-SOMMER, ZS., SHEPHERD, N., TACKE, E., GIERL, A., ROHDE, W., LECLERCQ, L., MATTES, M., BERNDTGEN, R., PETERSON, P. A. & SAEDLER, H. (1985). Influence of transposable elements on the structure and function of the A1 gene of *Zea mays*. *EMBO Journal* **6**, 287–294.

STEVEN, A. C., TRUS, B. L., PUTZ, C. & WURTZ, M. (1981). The molecular organization of beet necrotic yellow vein virus. *Virology* **113**, 428–438.

WALKER, J. E., SARASTE, M., RUNSWICK, M. J. & GAY, N. J. (1982). Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal* **1**, 945–951.

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