A Comparison of Three Potyviruses by Direct Hybridization Analysis

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Appreciation is expressed to P. Palukaitis for advice on direct molecular hybridization and for providing Taylor primer DNA. Special appreciation is expressed to J. S. Cole of the Biological Carcinogenesis Branch of the National Cancer Institute and J. W. Beard of Life Science, Inc., Gulfport, FL 33707, for supplying avian myeloblastosis reverse transcriptase.

Appreciation is extended to R. H. Baum for the single-lesion isolates of use in this study, to M. Collins, B. Sammons, and T. Zimmerman for purified blackeye cowpea mosaic, watermelon mosaic 1 and 2, and tobacco mosaic viruses, respectively, and to P. M. Burrows for analysis of melting profiles and R_0t kinetics.

Research in support of Regional Research Project S-127, Forage Legume Viruses. Technical Contribution 2061 of the South Carolina Agricultural Experiment Station, Clemson University.

Accepted for publication 11 April 1983.

ABSTRACT

Reddick, B. B., and Barnett, O. W. 1983. A comparison of three potyviruses by direct hybridization analysis. Phytopathology 73: 1506-1510.

Viral RNAs extracted from several potyviruses (bean yellow mosaic [BYMV-Scott], pea mosaic [PMV-204-1 and PMV-Pratt], and clover yellow vein [CYVV-C-81 and CYVV-Pratt]) were compared by using direct molecular hybridization. Complementary DNA was synthesized from the RNA templates with reverse transcriptase, and hybridization of the various RNA:cDNA combinations was analyzed. The two CYVV isolates and two PMV isolates showed partial sequence homology to the BYMV-Scott isolate. Partial sequence homology between PMV-204-1 and PMV-Pratt or between CYVV-C-81 and CYVV-Pratt was demonstrated; however, no sequence homology was found when either of the PMV isolates was compared with either of the CYVV isolates. In the cytoplasmic inclusion body subdivision II, several viruses are distinguished by the formation of nuclear crystalline inclusions. BYMV, PMV, and CYVV should be included in this category as different, but related, viruses on the basis of these cDNA data and previous biological and biochemical data.

The potyvirus group is the largest and probably the most economically important group of plant viruses (10). These viruses occur world-wide and cause diseases of agronomically important crops, including several important diseases caused by the Solanaceae and Leguminoseae (25).

At best, virus classification within the group is difficult; standard virological methods such as host range and symptomatology, particle morphology, physical properties, and serological tests seldom differentiate these viruses. Host range and symptomatology can vary with environmental conditions, cultivar, and time of year and it is often difficult to compare results obtained by different laboratories. Particle length can be quite variable for most members of the group. Serological tests of viruses within the group often show relatedness between viruses which differ in other properties (5,6,10,21). Edwardson (10) has classified the potyviruses into three subdivisions based on the type of virus-induced cytoplasmic inclusion bodies formed in host tissue. Bean yellow mosaic (BYMV), clover yellow vein (CYVV), and pea mosaic (PMV) viruses are in subdivision II and form nuclear inclusions in some of their hosts (5,10). Serological comparisons show the three viruses to be related, but not identical (5,18). Some investigators consider BYMV and PMV as separate viruses, but many consider them to be synonymous (3,18,24). Similarly, some investigators consider BYMV and CYVV to be synonymous while others consider them to be separate viruses (6,7,18,21).

There is a growing need for a more exact classification scheme within the potyvirus group. The purpose of this study was to further elucidate the relationship between BYMV, PMV, and CYVV by a comparison of their nucleotide sequence homologies using molecular hybridization analysis.

MATERIALS AND METHODS

Virus isolates and propagation. All BYMV, PMV, and CYVV isolates were propagated in *Pisum sativum* L. 'Dwarf Gray Sugar.' Isolates PMV-Pratt and CYVV-Pratt were received from M. J. Pratt. Isolate PMV-204-1 was that of S. Diachun. (The two PMV isolates have been designated as BYMV in previous publications.) Isolate BYMV-Scott was received from R. O. Hampton, and CYVV-C-81 was isolated in South Carolina. Watermelon mosaic virus (WMV) 1 and 2, received from D. E. Purcifull, were propagated in *Cucurbita pepo* L. 'Small Sugar'; blackeye cowpea mosaic virus (BlCMV) from F. W. Zettler, was propagated in *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye'; and our own soybean mosaic virus (SoyMV) isolated in Wisconsin was propagated in *Glycine max* (L.) Merr. 'Bragg.' Single lesion isolates of all BYMV, PMV, and CYVV strains were maintained in cultivar Dwarf Gray Sugar pea.

Virus purification. Two purification schemes were used to purify PMV, BYMV, CYVV, SoyMV, and WMV 1 and 2. Method 1 differed from that of Jones (17) by the addition of 0.05 M sodium diethyldithiocarbamate to the grinding buffer, precipitation by addition of polyethylene glycol (PEG 8000) to 4%, w/v, in the presence of 0.25 M NaCl followed by pelleting the virus through a 30% sucrose cushion, one-third volume of the tube, (66,000 *g* for 3 hr) prior to centrifugation in a 10–40% linear sucrose gradient. Method 2 was a further modification of method 1 (2). After the initial chloroform clarification and PEG concentration, the partially purified virus was treated with Triton X-100, 1% for 2 hr, subject to a second PEG precipitation followed immediately by equilibrium centrifugation (73,500 *g* for 15–18 hr) in cesium sulfate (0.75 ml of 53% wt/wt Ca_2SO_4 overlayered with 0.75 g Ca_2SO_4 in 3.75 ml of virus suspension). Virus was removed from cesium sulfate after a 10-fold dilution and concentration by high-speed centrifugation (229,400 *g* for 45 min). BlCMV was purified by the method of Lima et al (20). TMV was purified according to Khalil (19). Potyvirus concentrations were determined spectrophotometrically by using an extinction coefficient of 2.4 $(mg/ml)^{-1}cm^{-1}$ at 260 nm after correcting for light scattering.

RNA extraction. Extraction of RNA from each potyvirus was by the method of Brakke and Van Pelt (8). Disrupted virus was immediately layered onto 7.5–30% sucrose step gradient columns made in 0.5 M tris-HCl buffer, pH 9.0, and subjected to centrifugation at 81,500 *g* for 11–13 hr at 14 C. The RNA band was collected and precipitated with ethanol. After centrifugation the RNA pellets were resuspended in a small volume of H_2O and the

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concentrations were determined spectrophotometrically. The RNA was either used immediately or lyophilized and stored at −20 C.

TMV RNA was isolated by the method of Bruening et al (9), and healthy pea nucleic acid was extracted by the method of Jackson et al (16).

Gel electrophoresis. Viral RNAs were separated by electrophoresis according to Bruening et al (9). Tube gels (0.6 × 9 cm) were 0.5% agarose, 1.8% polyacrylamide, and 0.2% SDS; the electrophoresis buffer was 40 mM tris, 20 mM sodium acetate, pH 7.3, + 0.8 mM Na_2EDTA + 2% SDS. The gels were subjected to 5mA/gel for 2.5–3.5 hr and then scanned at 260 nm. A plot of relative mobility versus log molecular weight of TMV RNA and total *E. coli* RNA (Miles RNA markers; Elkhart, IN 46515) was used to estimate viral RNA molecular weights.

Synthesis of complementary DNA (cDNA). Synthesis of ^{3}H-cDNA was essentially by the method of Gould and Symons (12). Purified RNA (2 μg/50 μl of reaction mixture) of a BYMV, PMV, or CYVV isolate was added to 50 μl of reaction mixture containing 50 mM tris-HCl, pH 8.3, 8 mM dithioerythritol, 100 mM KCl, 8 mM $MgCl_2$, 0.50 mM dATP, dGTP, and dCTP, 3 nmol of ^{3}H-dTTp (sp act, 20.2 Ci/ml at 1 μg/μl) (New England Nuclear, Boston, MA 02118), 100 μg of actinomycin D per milliliter, 125 μg of Taylor primer DNA (23), and 100 units of avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated for 2.0 hr at 37 C. The reaction was stopped by the addition of 125 μl of H_2O, 5 μl of 5% SDS, and 20 μl of 3 N NaOH and incubated overnight at room temperature to hydrolize the RNA template. The cDNA was then fractionated on a 1.5 × 16-cm column of Sephadex G-50 (coarse) equilibrated with 0.1 M NH_4HCO_3. The cDNA fractions were pooled and freeze-dried after the addition of 100 μl of triethylamine per 1 ml of pooled cDNA.

Hybridization of cDNAs to RNAs. The freeze-dried cDNA was dissolved in either H_2O or "high-salt" or "low-salt" hybridization buffer (HSHB or LSHB) (0.10 M tris-HCl, pH 7.0, 1 mM EDTA, 0.05% SDS + either 0.18 m NaCl for "low salt" or 0.54 M NaCl for "high salt" hybridization buffer) to a dilution that contained the equivalent of 2×10^3 counts per min (cpm)/μl (11). The RNAs were appropriately diluted to give a specific R_0t value (12) in either HSHB or LSHB and 2 μl of cDNA were added (RNA:cDNA, 50:1). The hybridization solution was then boiled for 2–7 min in either sealed siliconized capillary tubes (100 μl) or 500 μl microfuge tubes and then incubated at 50 or 60 C for the appropriate length of time. The hybridization reaction was ended by chilling the tubes on ice.

S1 nuclease assay for extent of hybrid formation. The hybridization reaction mixture (40 μl) was diluted with 400 μl of S1 nuclease assay buffer (30 mM sodium acetate, pH 4.6, + 1 mM $ZnSO_4$ + 5% glycerol + 0.54 M NaCl). After mixing, two samples each of 200 μl were removed and placed in separate microfuge tubes. S1 nuclease (20–75 units per milliliter) was added to one tube and both tubes were incubated for 30 min at 45 C. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA) and 75 μg of bovine serum albumin to both tubes. After 30 min or more on ice, the TCA-precipitated material was collected on Whatman GF/A filters, washed thoroughly with cold 5% TCA and once with absolute ethanol. Radioactivity of the precipitate was determined by liquid scintillation spectrometry. The amount of hybrid resistant to S1 nuclease was calculated by dividing the cpm of the S1 nuclease-treated sample by the cpm of the untreated sample. The values were corrected for S1 nuclease resistance of the cDNA and standardized by using Gonda and Symons' formula (11).

Melting profiles. Homologous and heterologous hybridization reaction mixtures (100 μl) were incubated at 50 C to an R_0t value exceeding 1.0 mol·sec^{-1}. After chilling, the mixtures were placed in a water bath in which the temperature was raised 1 C/min. At 5-C intervals, 10-μl aliquots were removed, S1 nuclease-digested, and the percent hybridization was determined as previously described (22).

RESULTS

Virus purification. Bean yellow mosaic virus, PMV, and CYVV isolates that were purified by using Method 1 yielded 2–5 mg of virus per 100 g of infected pea tissue, except during summer months. The absorbance (A) ratio 260 nm/280 nm was 1.15–1.23 (corrected for light scattering). Virus purification using Method 2 yielded 4–20 mg of virus per 100 g of infected tissue with an $A_{260\ 280}$ value of 1.17–1.23 (corrected).

RNA extraction. In most RNA preparations density gradient centrifugation revealed a single homogenous absorbance peak of RNA (estimated molecular weight of 3×10^6 daltons) which migrated as a single band in analytical polyacrylamide-agarose gels. This molecular weight is in general agreement with those of other potyviruses (13,15); however, degradation of the RNA was observed with several RNA preparations. When degradation was detected, the RNA was not used for further tests. Undegraded RNA was infectious and was considered acceptable for use in hybridization studies if single homogenous bands were found in both density gradient centrifugation and gel electrophoresis.

Direct hybridization. Salt concentrations of 0.54 M or 0.18 M NaCl, in either the hybridization or S1 nuclease buffer, had no effect on estimations of sequence homology for the homologous hybrids. Hybridization reactions carried out at 50 C, as opposed to 60 C, had a lower background of S1 nuclease resistance (2–7% at 50 C, 10–14% at 60 C). S1 nuclease concentrations of 25 and 75 units per milliliter were tested under "high-salt" conditions. A concentration of 75 units of S1 nuclease per milliliter gave the same estimate of sequence homology between homologous cDNA and RNA, but a lower background of S1 nuclease resistance. Reaction conditions for all further tests were 50 C and 75 units S1 nuclease per milliliter to reduce the S1 nuclease-resistant background and

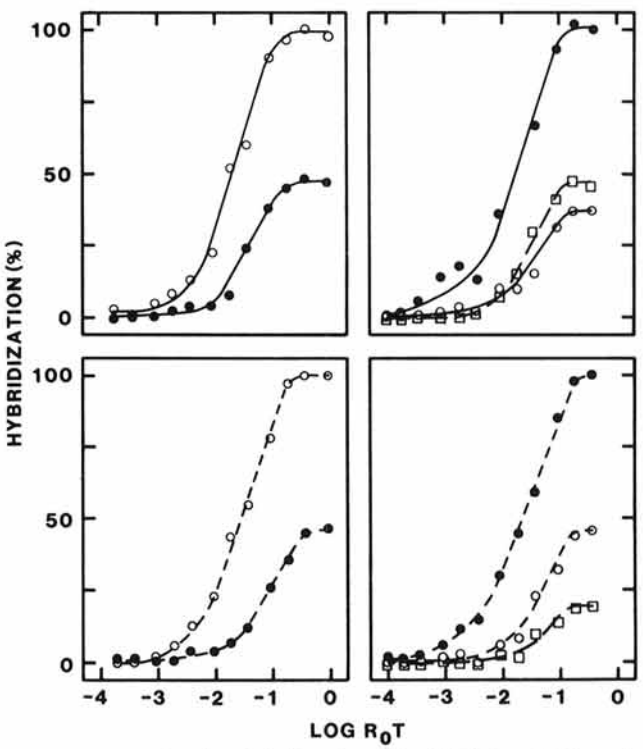

Fig. 1. R_0t curves showing hybridization of **A,** PMV-Pratt RNA against PMV-Pratt cDNA (O——O) and PMV-204-1 cDNA (•——•); **B,** PMV-204-1 RNA against PMV-204-1 cDNA (•——•); BYMV-Scott cDNA (□——□), and PMV-Pratt cDNA (O——O); **C,** CYVV-C-81 RNA against CYVV-C-81 cDNA (O——O), and CYVV-Pratt cDNA (•——•); and **D,** CYVV-Pratt RNA against CYVV-Pratt cDNA (•——•), CYVV-C-81 cDNA (O——O), and BYMV-Scott cDNA (□——□). Values are corrected for S1 nuclease resistance of the cDNAs and curves are standardized to 100%. Curves represent least square fits as described in the text.

"high-salt" concentrations, 0.54 M NaCl, in both hybridization and S1 nuclease steps to detect even imperfect sequence homology.

Hybridization kinetic studies were conducted to characterize the RNA:cDNA combinations only for those virus isolates and cDNAs that showed some degree of sequence homology in initial tests (*unpublished*). The homologous kinetic curves (Fig. 1) show a smooth, sharp, single-phase response with no rapidly annealing fractions. The theoretical formulation for fraction of nucleic acid strands renatured (f) at time t (4) can be expressed in the form: $f/g = 1 - 1/(1 + R_0t/h)$. The data were fitted to this relationship by least squares analysis to obtain estimates of the upper asymptotes (g) and $R_0t_{½}$ (h). In all cases, the data coincided with the best-fit curves (Fig. 1). The $R_0t_{½}$ values reflect the RNA sequence complexity which is related to the molecular weight of the RNAs used as templates for the cDNA synthesis. It has been shown that the ratio of $R_0t_{½}$ is a constant (4,12). Values obtained for this constant (Table 1) are similar to values obtained with other viruses (11).

The cDNAs of PMV-204-1 and PMV-Pratt, BYMV-Scott, CYVV-C-81, and CYVV-Pratt were allowed to anneal with several RNAs (Table 2) to an R_0t value exceeding their $R_0t_{½}$ by at least 100-fold. Actual percent hybridization figures were 50–70% for homologous reactions. All the reciprocal combinations (ie, PMV-204-1 cDNA with PMV-Pratt RNA and PMV-Pratt cDNA with PMV-204-1 RNA) gave the same estimate of sequence homology, within experimental error. As indicated in the initial test, little or no sequence homology was found between RNA from PMV-204-1 or PMV-Pratt when tested against cDNA to CYVV-C-81 or CYVV-Pratt and vice versa. However, sequence homology between PMV-204-1 and PMV-Pratt was found and hybrids also formed between CYVV-C-81 and CYVV-Pratt. BYMV-Scott RNA or cDNA hybridized to all four of these PMV and CYVV isolates. When the percent sequence homologies for all heterologous combinations were compared, BYMV-Scott differed from all other genomes examined except when PMV-204-1/PMV-Pratt was compared with PMV-204-1/BYMV-Scott. Thus BYMV-Scott may be more closely related to the PMV isolates, especially PMV-204-1, than to the CYVV isolates, but BYMV-Scott is definitely different from both PMV and CYVV.

Low levels of sequence homology were found in hybridization reactions of BYMV, PMV, or CYVV cDNA to other potyvirus RNAs (Table 2). No sequence homology was seen with TMV RNA and very little with healthy plant RNA.

Thermal denaturation curves of homologous hybrids showed a smooth, sharp transition indicating little base-pair mismatching with a Tm around 90 C (Table 3). The heterologous hybrids showed a broader denaturation curve with the Tm being much lower (75–80 C) indicating considerable base-pair mismatching.

DISCUSSION

Two methods of virus purification were used throughout this study. Both methods gave a clean virus preparation as indicated by visual inspection of the last high speed centrifugation pellet and spectrophotometric data. Method 2 had a four to fivefold increase in yield when compared to Method 1, because much virus was lost in the sucrose density gradient centrifugation step due to

TABLE 1. Hybridization kinetic data of BYMV, PMV, and CYVV isolates

RNA	cDNA	Replication/ R_0t value	Maximum hybridization[a,d] (%)	$R_0t_{½}$[b] (10^{-2} mol·sec^{-1})	$R_0t_{½}$/ MW[c] of ($\times 10^{-9}$)
PMV-204-1	PMV-204-1	4	100 ± 4	1.8 ± 0.3	6.0
	PMV-Pratt	6	44 ± 3	5.8 ± 1.2	
	BYMV-Scott	4	54 ± 3	4.0 ± 0.7	
PMV-Pratt	PMV-Pratt	4	100 ± 3	2.4 ± 0.3	8.0
	PMV-204-1	4	51 ± 3	5.4 ± 1.1	
CYVV-C-81	CYVV-C-81	4	100 ± 2	3.0 ± 1.2	10.0
	CYVV-Pratt	4	50 ± 2	9.7 ± 1.2	
CYVV-Pratt	CYVV-Pratt	4	100 ± 2	2.8 ± 0.2	9.3
	CYVV-C-81	4	51 ± 3	6.6 ± 1.1	
	BYMV-Scott	4	23 ± 2	7.5 ± 1.8	

[a] Upper asymptote (g) adjusted to 100% for homologous reactions; ± standard error.
[b] $R_0t_{½}$; ± standard error. These values were not corrected for cDNA lengths or G + C content.
[c] Molecular weight of all RNAs assumed to be 3×10^6 daltons.
[d] Uncorrected percentages for homologous hybridizations were: PMV-204-1, 74%; PMV-Pratt, 63%; CYVV-C-81, 82%; CYVV-Pratt, 71%.

TABLE 2. Estimated percentage sequence homology of BYMV and CYVV RNA

RNA used in hybridization reaction	RNA used for cDNA preparation				
	PMV-204-1	PMV-Pratt	BYMV-Scott	CYVV-C-81	CYVV-Pratt
PMV-204-1[a]	100[b,d]	55.2 ± 3.2	48.6 ± 2.7	ND[c]	ND
PMV-Pratt[a]	55.1 ± 2.7	100	22.8 ± 2.7	ND	9.4 ± 2.8
BYMV-Scott[a]	46.5 ± 3.1	31.0 ± 3.2	100	24.4 ± 2.4	23.4 ± 2.8
CYVV -C-81[a]	ND	ND	21.4 ± 2.7	100	46.9 ± 2.8
CYVV-Pratt[a]	ND	0.004 ± 3.2	20.0 ± 2.7	42.3 ± 2.4	100
WMV 1	0	13	4	0	9
WMV 2	0	0	13	0	12
SoyMV	5	7	9	6	4
BICMV	7	13	3	3	12
TMV	0	0	0	0	0
Healthy plant RNA	0	4	4	4	3

[a] Isolates more rigorously tested (R_0t curves, Tm, and reciprocal RNA:cDNA tests standard error).
[b] Reactions corrected for S1 nuclease resistance and standardized (0–100%).
[c] Not different from zero homology within experimental error.
[d] Actual percent hybridization of homologous reactions: PMV-204-1, 74%; PMV-Pratt, 63%; BYMV-Scott, 73%; CYVV-C-81, 82%; CYVV-Pratt, 71%.

aggregation with Method 1. RNA extracted from most virion preparations purified by either Method 1 or 2 often yielded a single band (MW 3×10^6 daltons) both in the sucrose density gradient and after gel electrophoresis; however, Method 1 yielded undegraded RNA more often than Method 2. The RNA was also shown to be infective on *C. amaranticolor* in half-leaf infectivity tests. Thus, virus yield and purity were optimized, and RNA extracted from purified virus was homogeneous and infective.

Purified RNA was then used as a template for cDNA synthesis. cDNA against cucumber mosaic virus RNA prepared by this method is representative of the whole genome from which it is transcribed, is not enriched in any one sequence, and the midpoint of the homologous kinetic curve ($R_0t_{½}$) is proportional to the molecular weight of the RNA (12). Hybridization kinetic tests and the specificity of the cDNAs indicate that the cDNAs that we obtained are representative of the respective RNAs from which they were transcribed and we assume they are not enriched in one particular region. The heterologous kinetic curves show a $R_ot_{½}$ value which is somewhat slower than the homologous reaction in all cases (Fig. 1). For instance, the heterologous reaction $R_ot_{½}$ (4.3×10^{-2} mol·sec^{-1}) between cDNA to CYVV-C-81 and RNA to CYVV-Pratt was two times slower than the homologous reaction (cDNA to C-81 with RNA to C-81) $R_0t_{½}$ (2.5×10^{-2} mol·sec^{-1}). Since none of the heterologous reactions continued to 100% even at high $R_0t_{½}$ values, contamination cannot be the explanation for the shift of $R_{0't½}$. A possible explanation could be that the heterologous hybrids have a sufficiently different melting temperature to cause this effect. Heterologous hybrids showed only partial hybridization, and were slower in forming than were the homologous hybrids, and base-pair mismatching was evident. With this in mind, careful interpretation must be made of the data found in Table 2. Because of base-pair mismatching, the actual percentage hybridization values of heterologous combinations in the table are probably too high. However, it is felt that valid relationships can be inferred from the data. For example, CYVV-C-81 and CYVV-Pratt have a high degree of sequence homology; however, they have no apparent relationship with PMV-204-1 or PMV-Pratt, which have substantial sequence homology to each other. All four of these isolates show varying degrees of sequence homology with BYMV-Scott.

The results of Abu-Samah and Randles (1) were similar to ours in that pseudo-first-order kinetics for homologous cDNA-RNA reactions with three BYMV strains were obtained and salt concentration had little effect on S_1 nuclease resistance of the hybrids. Moreover, heterologous sequence homologies among their strains of BYMV were well below the homologous values. Preliminary hybridization data (O. W. Barnett, *unpublished*) indicates that BYMV strains -G, -Q, and -S (1) are more closely related to BYMV-Scott than to either PMV or CYVV. The PMV strain used by Abu-Samah and Randles (1) was not the same as the PMV strains used in this study; further work is needed to establish the relationship of this PMV isolate with those used here. For each cDNA, the hybridization reaction with homologous RNA was faster ($R_0t_{½}$) than the heterologous reaction(s), but Abu-Samah and Randles (1) found the same rates for both types of reactions.

Our data suggest the possibility that CYVV, BYMV, and PMV form an evolutionary continuum in relationship to each other.

TABLE 3. Melting temperatures[a] of BYMV, PMV, and CYVV hybrids

RNA	cDNA	Tm (C)
PMV-204-1	PMV-204-1	90.0 ± 0.1
PMV-204-1	PMV-Pratt	80.4 ± 2.1
PMV-Pratt	PMV-Pratt	89.9 ± 0.1
BYMV-Scott	BYMV-Scott	90.4 ± 0.2
CYVV-C-81	CYVV-C-81	90.0 ± 0.05
CYVV-Pratt	CYVV-C-81	75.9 ± 1.9
CYVV-Pratt	CYVV-Pratt	90.0 ± 0.03

[a] Defined as that temperature at which 50% of the hybridized form is retained (estimates from median points of fitted logistic curves, ± standard error).

Jones and Diachun (18) suggested this relationship on the basis of host range, symptomatology, and serological data, and call all isolates BYMV instead of dividing them among BYMV, PMV, and CYVV. Others suggest there are enough biological (5) and translation product (14) differences to separate BYMV and CYVV. Molecular hybridization is not the final solution to this question of relationships; however, the data obtained from this study indicate that PMV is no more closely related to BYMV than is CYVV. Thus, if CYVV is considered to be a separate virus (as presently considered by most research workers) then PMV also must be considered a separate virus and not a strain of BYMV.

The previous serological, host range, and in vitro translation data and hybridization data, as well as the RNA:cDNA relationships from this work, are in agreement with the following proposal: BYMV, PMV, and CYVV are different, but related, viruses belonging to the category of viruses which form nuclear crystalline inclusions in the cytoplasmic inclusion body subdivision II and each of these viruses has many strains.

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