



Purification and characterization of a Montana RMV-like isolate of barley yellow dwarf virus
by Susan Mary Geske

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Plant Pathology

Montana State University

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Abstract:

A Montana barley yellow dwarf virus (BYDV) isolate, designated MT-RMV-V, was serologically indistinct from the NY-RMV type. It could be distinguished only by differential aphid vector transmission. Diameters of purified virions averaged 24.7 nm (S.D.=1.2 nm, n=559). Nucleic acid size was estimated by denaturing and non-denaturing gel electrophoresis with ethidium bromide staining. The relative molecular weight of the RNA was 1.7×10^6 . The coat protein size was 21.7 Kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie blue staining. A cytological investigation of infected phloem cells by electron microscopy revealed that MT-RMV-V induced alterations were similar to those of a Manitoba RMV isolate and to those of an RPV isolate.

Use of 1-fold, 2-fold and 16-fold degenerate primers derived from published sequences of potato leaf roll virus (PLRV), beet western yellows virus (BWYV) and an Australian BYDV-PAV isolate in polymerase chain reactions generated DNA fragments of the following approximate lengths: 370 bp, 520 bp and 1.4 kb. The smallest fragment encompassed part of the 3' end of the RNA dependent RNA polymerase gene. The middle sized fragment encompassed most of the viral coat protein gene, while the largest fragment encompassed both regions and the intervening non-coding region. The 520 bp fragment was compared by restriction enzyme analysis with similarly obtained fragments from BWYV and five NY BYDV isolates. Unique banding patterns distinguished MT-RMV-V from the others. The 520 bp fragment from MT-RMV-V and NY-RMV and the 370 bp fragment from MT-RMV-V were cloned using pUC19 or pUC119 vectors and were sequenced. There was 80% nucleotide sequence homology in the coat protein region between the MT and NY RMV isolates. This resulted in a 77% amino acid sequence homology. Both isolates shared greater nucleotide sequence homology with BWYV, PLRV and BYDV-RPV, in the coat protein region, than with the other BYDV isolates. The MT-RMV-V and NY-RMV isolates should either be classified as strains of BWYV or should be reclassified, along with the NY-RPV isolate, as a separate virus from BYDV.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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LIST OF ABBREVIATIONS

- BLRV - Bean leafroll virus
- BMV - Brome mosaic virus
- BWYV - Beet western yellows virus
- BYDV - Barley yellow dwarf virus
- DEPC - diethyl pyrocarbonate
- DNA - deoxyribonucleic acid
- Lu1 - 5'-CCAGTGGTTRTGGTC-3' oligonucleotide DNA primer
- Lu4 - 5'-GTCTACCTATTTGG-3' oligonucleotide DNA primer
- MES - 2-[N-Morpholino]ethanesulfonic acid
- MT-RMV-V - Montana BYDV-RMV isolate from Valier, Montana
- NY-MAV - New York BYDV isolate vectored by Sitobian avenae (Fabr.)
- NY-PAV - New York BYDV isolate vectored by Rhopalosiphum padi (L.) and S. avenae
- NY-RMV - New York BYDV isolate vectored by Rhopalosiphum maidis (Fitch)
- NY-RPV - New York BYDV isolate vectored by R. padi
- NY-SGV - New York BYDV isolate vectored by Schizaphis graminum (Rondani)
- PLRV - Potato leafroll virus
- Pol1 - 5'-CGACTGCAGGGNTTYGAYTGG-3' oligonucleotide DNA primer
- Pol2 - 5'-GCTGGATCCTGNSWRCARAAAYTC-3' oligonucleotide DNA primer
- RNA - Ribonucleic acid
- TMV - Tobacco mosaic virus

ABSTRACT

A Montana barley yellow dwarf virus (BYDV) isolate, designated MT-RMV-V, was serologically indistinct from the NY-RMV type. It could be distinguished only by differential aphid vector transmission. Diameters of purified virions averaged 24.7 nm (S.D.=1.2 nm, n=559). Nucleic acid size was estimated by denaturing and non-denaturing gel electrophoresis with ethidium bromide staining. The relative molecular weight of the RNA was 1.7×10^6 . The coat protein size was 21.7 Kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie blue staining. A cytological investigation of infected phloem cells by electron microscopy revealed that MT-RMV-V induced alterations were similar to those of a Manitoba RMV isolate and to those of an RPV isolate.

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CHAPTER 1

INTRODUCTION

Presently, barley yellow dwarf virus (BYDV) is the type member of the Luteovirus group. Members of this group are characterized by having isometric virions and positive sense single stranded RNA. Their capsid is composed of protein sub-units of a single molecular species. The viruses are phloem limited and are often found in low concentration in host plants (5,11,16,17,26,54,58,64,78,126,138,144,145). Members generally cause yellowing symptoms in the host, hence the name 'Luteo' for this group. All members are usually transmitted by aphids in a circulative, persistent, but non-propagative manner (17,39,42,78,144,148).

BYDV is found throughout the world in cereal growing regions, and is restricted to monocotyledonous plants. Other important Luteoviruses include beet western yellows virus (BWYV), a widespread virus infecting many dicotyledonous host species; potato leafroll virus (PLRV), found world wide but generally restricted to potatoes; and bean leafroll virus (BLRV) which normally infects legumes. A variety of strains have been associated with each of these members. In the past, viruses have been named by the host plant in which they were originally found. Because of this

naming system, synonymous names for a single virus are all too common. This has led to an ever increasing number of viruses in the luteovirus group. Casper (17) tried to narrow this list in 1988 by aligning like strains and viruses on the basis of serological relationships.

BYDV is the causal agent of a leaf discoloring and stunting disease of cereals that was first described in 1951 by Oswald and Houston in California (87). Five strains, or variants (49,50,101,112,117,119,120,121) or more recently isolates (113), of this virus have been reported. They were initially differentiated and named for the predominant aphid species which could vector them. The strains and their respective vector(s) are:

PAV - Rhopalosiphum padi (L.), Sitobion avenae (Fabr.)

MAV - S. avenae

SGV - Schizaphis graminum (Rondani)

RPV - R. padi

RMV - Rhopalosiphum maidis (Fitch)

The PAV strain is an aphid non-specific strain. Both aphid species, noted above, are capable of transmitting this strain.

Aphid vector specificity has been considered to be one of the most important methods used to differentiate and classify recently collected BYDV isolates. Gildow (39,40,41,43,44,45) has studied the complex aphid-BYDV relationship extensively using the electron microscope. In

thin sections of aphids, he discovered the importance of membrane barriers relative to the internal circulation of BYDV isolates in the aphid. The initial barrier is the lumen membrane of the hindgut. However this membrane is not highly selective and most BYDV isolates can pass through it in any given aphid species. The plasmalemma of the accessory salivary gland appears to be responsible for the observed aphid-virus vector specificity. It is this latter membrane that determines if a BYDV isolate will be transmitted by a particular aphid species. In one study, Gildow examined virus-membrane selectivity using S. avenae and the MAV, PAV, and RPV isolates. He found that all three isolates could pass through the hindgut lumen membrane, but only MAV and PAV could pass through the accessory salivary gland plasmalemma. No detailed studies of the exact receptor site(s) on the plasmalemma have been reported. Nor have aphid virus transport studies using BYDV isolates which are vectored by a variety of non-related aphid species been reported.

Classification of the BYDV isolates was made in order to explain the observed similarities and differences within the group. The PAV, MAV, and SGV isolates, are considered to be in sub-group I and the RPV and RMV are considered to be in sub-group II. These initial groupings were made based on serology (50,107,110) and pathological ultrastructural changes induced in the host cells (45,47). Analyses of ds

RNA profiles (41,136,137) and nucleic acid sequences (141,142) have lent support to these divisions. The genomic organization of the six open reading frames appears to differ in the two sub-groups (68,78). The two overlapping non-structural genes are located within the 5' half of the genome for both sub-groups. But they encode 39 Kd and 60 Kd products for PAV isolates and 70 Kd and 69-72 Kd products for BWYV, PLRV and NY-RPV (78,141). In the PAV and MAV isolates the coat protein gene, with an internal virus-linked protein gene, is located more centrally in the genome. In RPV these genes are located more toward the 3' end of the genome. In addition, the putative protease gene is located at opposite ends of the genome for each sub-group. With PAV and MAV the protease gene is located at the 3' end, but in RPV it is at the 5' end.

BYDV can cause periodic epidemics resulting in significant crop loss (3,21,46,48,150). Often plants infected with a single BYDV isolate display physiological stress (2,29,30,31,33,36,63,65,66,67,73,129) which results in not only decreased yields, but increased plant susceptibility to other pathogens (130). Multiple infections of plants by two or more isolates usually reduce yields more than do single infections. Synergistic effects between the MAV and RPV isolates, as well as between the RMV and PAV isolates have been reported (46). In addition, one BYDV isolate may act as a helper virus to allow the

subsequent uptake of a non-transmitted isolate by a particular aphid species (103). The phenomena of helper viruses and heterologous encapsidation, ie. the coat protein of one isolate encapsidating the nucleic acid of another isolate, greatly enhances the possibility of increased spread of a more virulent, but perhaps less represented strain within a crop. W.F. Rochow observed that in NY grain crops, the prevalence of vector-specific isolates decreased while the prevalence of a vector non-specific isolate increased during a 10 year period (102,105,106,109,114,115). Although no other U.S. geographic region has been studied as extensively, the NY study suggests that BYDV isolate prevalence could change over time. For example, it is possible that a less virulent isolate could be displaced by a more virulent one in a region.

The most common control measures are: the use of selected planting dates, the use of tolerant cultivars and the application of pyrethroids and organophosphates to control aphids. Altering crop planting dates avoids having young, susceptible plants available to migrating, infective aphids (21,48,62,71,97,99,132,150). Breeding for resistance to BYDV has had limited success (56,93). Cereal cultivars which contain genes which condition for tolerance to BYDV are found in barley and oats, but these cultivars are often restricted in their growing regions. Some cereals are

naturally resistant to aphid feeding (84), but breeding experiments with these plant cultivars have been limited.

Because cereal crops are annual, the virus must be introduced to the plants each year by the aphids. It has been suggested that one host species may act as a reservoir of the virus for secondary spread (13). Corn was suggested as one such possible reservoir. Brown et al. (13) suggested that a Washington PAV isolate may move from wheat to corn via S. avenae and then from corn to wheat via R. padi. However, studies by Blackman et al. (9,10) and Brown and Blackman (14) suggest that movement from one crop to another, such as from corn to barley, by corn leaf aphids (R. maidis) is not probable. V.F. Eastop and R.L. Blackman (British Museum of Natural History, London England) analyzed the karyotype of corn leaf aphids collected from corn, spring barley and wheat. They determined that aphids collected from spring barley had a $2n=10$ karyotype whereas aphids obtained from corn had a $2n=8$ karyotype. Both of these aphid types were capable of transmitting BYDV RMV-like isolates efficiently (15). A clone of W.F. Rochow's NY biotype of R. maidis had a $2n=8$ karyotype, indicating that it was probably originally collected from corn. A $2n=8+1$ karyotype was reported for aphids collected from wheat plants in some parts of the world (10). It has not been reported that the three distinct aphids can switch their feeding from corn to spring barley or to wheat in nature

(9,15). This finding would eliminate one possible mechanism by which BYDV can overwinter and spread between two different crops.

The determination of multiple karyotypes for individual aphid species has been reported previously by Blackman (10). Often the chromosome change does not result in a gross morphological change in the aphid, and thus the splitting of one species into two or more species has not been recommended. However, it is unknown what genomic information is carried on the extra chromosome and if it has any influence on an aphid's ability to vector viruses. It is also unknown what, if any, genetic information is lost for aphids which have reduced chromosome numbers.

Yount and Carroll (149) reported the presence of serologically unique RMV-like isolates collected in Montana from 1978-1981. These original isolates failed to react to W.F. Rochow's bank of NY-BYDV antisera in double antibody sandwich enzyme-linked immunosorbent assay. This was a significant departure from results normally obtained for BYDV isolates. Generally field isolates react with an antiserum to at least one of the NY type isolates (20,22,23,25,27,32,59,89,107,108,134). Because of this serological difference, a more thorough investigation of MT RMV-like isolates was undertaken. During 1985 and 1986, a second set of BYDV field isolates was collected. Five RMV-like isolates, based on aphid transmission data, were

retained for further characterization. Three of the MT RMV-like isolates were efficiently transmitted by corn leaf aphids and by greenbugs (15). When these isolates were tested against NY polyclonal antisera, they reacted positively with NY-RMV immunoglobulin but not with NY-SGV, NY-RPV, NY-PAV or NY-MAV immunoglobulins in enzyme-linked immunosorbent assays (15). Whether the original RMV-like isolates present in Montana changed over time, or if new isolates were moved into the state by migrating aphids, or whether the antiserum produced recently is more effective at detecting RMV-like isolates is uncertain.

Because of the anomaly in aphid vectors for the MT RMV-like isolate, we hypothesized that other properties of the MT isolate may differ from those of the NY-RMV isolate. The purpose of this research was to purify one of the MT RMV-like isolates and characterize it chemically and physically. A second objective was to examine infected phloem cells for cytopathological alterations. A third objective was to produce antibodies specific for the MT RMV isolate. Our final objective was to partially sequence the coat protein gene, since this gene may be involved in aphid vector specificity. If the MT and NY isolates were to be dissimilar genomically, we hypothesized that it would be in the coat protein region. By characterizing an RMV-like isolate, we hoped to gain basic knowledge that could help

elucidate the taxonomic relationships and classification within the BYDV group.

CHAPTER 2

BIOLOGICAL INVESTIGATION

Introduction

Barley yellow dwarf virus (BYDV) is the causal agent of a dwarfing and leaf discoloring disease of gramineous hosts (87,120). Four aphid specific virus isolates and one aphid non-specific isolate of BYDV have been described by Rochow (113,118), Gill (49,50), and Rochow and Muller (111). Since that time, most workers worldwide have compared their local field isolates of BYDV to the five New York (NY) isolates of Rochow (1,13,27,77,94,112,131). Although some of those local isolates were characterized and eventually designated according to the location in which they were collected; i.e. IL-PAV (24), P-PAV (55), and CA-RPV and CA-PAV (22), each was similar to at least one of the NY isolates. These similarities included, but were not limited to, ds RNA profiles, aphid vector specificity, serology and cross protection.

Five Montana (MT) BYDV isolates collected in 1986 were found to be RMV-like on the basis of vector specificity. Upon further investigation, it was determined that the MT RMV-like isolates were similar to the NY-RMV isolate

serologically, but were more virulent in Avena byzantina (Koch) cv. Coast Black oats than either NY-RMV (15). Additionally, the MT isolates were transmitted by both Rhopalosiphum maidis (Fitch) (corn leaf aphid) and Schizaphis graminum (Rondani) (greenbug). Two MT biotypes of R. maidis, one having a karyotype of $2n=10$ and the other having a karyotype of $2n=8$, vectored three MT RMV-like isolates with average transmission efficiencies of 41% and 59% respectively (9,15). By comparison, the NY biotype of R. maidis, having karyotype $2n=8$, had only an average transmission efficiency of 29% for the three MT RMV-like isolates. More importantly, the NY biotype of S. graminum had a relatively high average transmission efficiency of 19% with the same three MT RMV-like isolates (15). S. graminum rarely transmits NY-RMV (16).

Although it is unusual for two serologically indistinct BYDV isolates to have different aphid transmission properties, it is not without precedent. Creamer and Falk (22) reported a California (CA) RPV isolate which was serologically indistinct from the NY-RPV type, and yet, was non-specifically transmitted by three aphid species. NY biotypes of Rhopalosiphum padi (L.) (oat-birdcherry aphid) and Sitobion avenae (Fabr.) (English grain aphid) as well as CA biotypes of S. avenae and S. graminum were able to vector the CA-RPV isolate.

Research devoted to RMV isolates has been limited mostly to aphid transmission, cross protection and various serological investigations. Research on the purification and characterization of RMV virions was needed in order to advance the basic understanding of these isolates. The purpose of this study was to purify and characterize one of the MT RMV-like isolates. A cytological investigation of infected oat leaves was undertaken to characterize one of the many biological properties of this isolate. A final objective was to develop MT-RMV specific polyclonal and monoclonal antibodies for use in diagnostic testing.

Materials and Methods

Host Plant

Three seeds per 10 cm plastic pot of Avena byzantina (Koch) cv. Coast Black oats were sown in soilless Sunshine mix and maintained for two weeks prior to inoculation in a Percival growth chamber, programmed for a 12 hr photoperiod (10,000 lux) and 24 C temperature. Plants were watered every two days and were fertilized with an all-purpose commercial fertilizer every two weeks.

Aphids

Rhopalosiphum maidis (corn leaf aphid) colonies were supplied by W.F. Rochow in 1986, or collected from spring

barley and corn fields in Montana during 1985 and 1986.

Colonies were designated as follows:

NY-RM (Derived from the NY biotype of R. maidis)

MT-RM SB (MT R. maidis from spring barley)

MT-RM C (MT R. maidis from corn)

Non-viruliferous colonies were increased on Hordeum vulgare (L.) cv. Klages within nylon mesh cages. Cages were 13 cm in diameter and 50 cm high. Aphid colonies were kept in a Conviron growth chamber programmed for 20 C and 15 hr photoperiod (10,000 lux). New colonies were started every month with first instar nymphs from an old colony or from three to five isolated, apterous adults. Six to twelve aphids were used for each pot containing 50 young Klages barley plants.

Virus

An RMV-like isolate of BYDV was obtained in 1986 from infective aphids feeding on spring barley located near Valier, MT. This isolate was subsequently characterized on the basis of its aphid transmission and serological characteristics (15). It has been maintained by serial aphid transfer in California Red oats (A. sativa) and Coast Black oats. This BYDV isolate has been designated as MT-RMV-V. The RMV isolate from W.F. Rochow's New York collection, designated NY-RMV, was obtained in 1986 and was similarly maintained.

The MT-RMV-V isolate was propagated by allowing healthy, nonviruliferous aphids to feed on detached, MT-RMV-V infected oat leaves for one to two days followed by a five to six day inoculation access feeding on two to three week old healthy oat plants (116). In excess of 20 presumed infective aphids, per plant, were given the opportunity to feed. Plants were enclosed by 8 cm x 30 cm acrylic and nylon mesh cages to prevent escapes and cross contamination. Plants were placed in the green house programmed for 24 C and a 15 hr photoperiod (10,000 lux) or in a Conviron growth chamber programmed for 20 C and a 15 hr photoperiod. Plants were fumigated for 30 minutes with Vapona (Diclorvos, Diamond Shamrock Corp.) to kill the aphids. Fumigated plants were then returned to the green house or the growth chamber, where they developed symptoms three to five weeks after inoculation. These plants were either maintained as stock plants or were harvested and stored at -20 C.

Virus Purification and Assay

The Valier RMV isolate of BYDV was purified using a method modified from those described by Hammond et al. (55), D'Arcy et al. (24), Christie et al. (18), Lee and Davis (74) and S.Gray (personal communication). The buffer system used and a reduction in the number of low speed centrifugations were the major modifications in the purification method reported here. Generally, 50 g of starting material was

used, but as little as 35 g or as much as 100 g was used without having to alter the procedure or time involved for processing. All but completely discolored and dried leaves were harvested from diseased plants for virus purification. Leaves, and occasionally roots and stems, were ground in excess liquid nitrogen with a mortar and pestle, then ground to a finer powder in a Sorvall Omni-Mixer. Cold buffer (0.1 M 4-morpholineethanesulfonic acid (MES), 0.02 M ethylenediaminetetraacetic acid (EDTA), 0.5% sodium sulfite, pH 6.2) was added 1:2 (w/v) and the mixture was homogenized for three to five minutes on ice. Extractase P20X enzyme (Finnsugar Biochemicals, Rochester, NY) was added at the rate of 0.02 g/ml mixture. After blending, the mixture was allowed to stand on ice 30 minutes and then 2.5 hr at room temperature. The mixture was given periodic homogenization for 15 seconds during this time. Triton X-100 was added at the rate of 5 ul per ml of total volume of homogenized mixture. The mixture was slowly stirred at room temperature for 30 minutes. A 1:1 mixture of cold chloroform and n-amyl alcohol was added to the homogenate at the rate of 3.3 ml per 5 ml of homogenate, blended rapidly for 30 seconds, and then stirred slowly at room temperature for 30 minutes.

This mixture was centrifuged in a Sorvall SS34 rotor at 12,100 x g for ten minutes, at 4 C. The supernatant was filtered through Kimwipe paper tissues. One-third volume of a 30% PEG (ave. mol. wt. 7000-9000), 0.6 M NaCl stock

solution was added to the supernatant. After shaking vigorously, the supernatant was allowed to stand overnight, at 4 C. The supernatant was centrifuged in a Sorvall SS34 rotor at 27,000 x g for ten minutes, at 4 C. Pellets were resuspended in 4 to 5 ml MES buffer, layered onto a 30% sucrose pad and centrifuged in a Beckman Ti80 rotor at 100,000 x g for 3 hr, at 4 C. Pellets were resuspended in 1 ml MES buffer for 1 hr and then layered on a sucrose gradient (4,12). The gradient was made by layering 8.0 ml each of 10, 20, 30, and 40% sucrose solutions in MES buffer and allowing the sucrose to diffuse at room temperature for 4 hr so the gradient would become continuous. The gradient was centrifuged in a Beckman SW28 rotor at 85,000 x g for 3 hr, at 4 C. After discarding the first 2 to 3 ml collected, four 8.0 ml fractions were collected, in sequence, from the top of the centrifuge tube with an Isco Model 640 fraction collector connected to an ISCO Model UA-5 absorbance monitor. Fraction F-1 was the first 8.0 ml collected, fraction F-2 was the second 8.0 ml collected, and so forth. Fractions were centrifuged in a Beckman Ti80 rotor at 150,000 x g for 3 hr, at 4 C. Each pellet was resuspended in 200 to 300 ul of buffer and assayed by ultraviolet absorbance spectroscopy to estimate virus concentration and purity and by transmission electron microscopy. Fractions were then stored at -20 C until further use.

Spectrophotometric readings of purified virus preparations were determined in a Beckman Model DU 50 spectrophotometer. Yields were estimated with the following formula and extinction coefficient of 8.0 supplied by S.M. Gray (personal communication).

$$\text{mg/ml} = [(A_{260} - A_{320}) \times \text{dilution factor}] / 8.0$$

Yields were then converted to mg/Kg of plant starting material. The average $A_{260/280}$ value was calculated, after correcting for light scattering (A_{320}) (4,28).

Formvar and carbon coated 300 mesh grids were prepared according to Christie et al. (18). Ten microliters of each centrifuge fraction (F-1, F-2, F-3 and F-4) from virus infected or healthy plant preparations were placed on individual grids for one to two minutes. Grids were washed with 1 ml filtered, distilled water containing 250 to 300 ug/ml bacitracin (Sigma), as a wetting agent, and then stained for 5 seconds with a 2.0% uranyl acetate solution in water which also contained 250 to 300 ug/ml bacitracin. After staining, grids were dabbed dry without further washing. Samples were observed at 60 kV with a Zeiss EM 10CA electron microscope. Virion measurements were taken from electron micrographs using a Zeiss Interactive Digital Analysis System (ZIDAS).

Estimation of Virion Chemical
Components

All Luteoviruses have been shown to be single stranded RNA viruses (17,78). Based on aphid specificity, disease symptoms, and virion morphological similarities between the MT-RMV-V isolate and the other BYDV isolates, the MT-RMV-V isolate was assumed to contain RNA. The size of the virion RNA was estimated by three different methods. In the first method, RNA was extracted from those fractions with the highest numbers of virions, as revealed by electron microscopic assay and A_{260} estimation of virion concentration. One-half volume of buffer (0.2 M glycine, pH 9.5, 0.2 M NaCl, 20 mM EDTA), 80 ul of 20% sodium dodecyl sulfate (SDS) (Gallard-Schlesinger, Carle Place, NY), and 16 ul of 20 mg/ml Proteinase K (Promega, Madison, WI) were added per milliliter of virion starting volume. The mixture was vortexed briefly and then incubated at 37 C for 30 min. Phenol was added at the rate of 2 ml/ml starting volume, and the mixture vortexed 1 minute before it was centrifuged at 12,000 x g for 30 min. at 4 C. RNA was precipitated from the aqueous phase with 0.1 volume of 3 M sodium acetate (NaOAc) and 3 volumes of cold, 100% ethanol (EtOH). The mixture was held at -70 C for 15 min. and then centrifuged at 12,100 x g for 30 min. at 4 C. The pellet was resuspended in 300 ul of 0.3 M NaOAc and RNA reprecipitated with 2.5 volumes of cold, 100% EtOH and stored overnight at -70 C. Viral RNA was

microfuged, washed, dried and resuspended in 20 ul of diethyl pyrocarbonate (DEPC) treated water as per accepted protocols (86,123,143). Virion RNA size was determined by 1% LE agarose (SeaPlaque, FMC BioProducts, Rockland, ME) electrophoresis after denaturation with formamide and formaldehyde (123). The entire 20 ul sample, approximately 21 ug, of resuspended RNA was loaded into each well. The gel was stained in 0.5 ug/ml DEPC treated water of ethidium bromide. A 0.24-9.5 Kb RNA ladder (BRL, Gaithersburg, MD), brome mosaic virus (BMV) RNA (Promega) and tobacco mosaic virus (TMV) RNA (52) were used as standards. BMV contains four nucleic acids in its virion. The relative molecular weights (Mr) of its RNA are Mr 1.0×10^6 , Mr 1.0×10^6 , Mr 0.7×10^6 and Mr 0.35×10^6 . TMV has a nucleic acid Mr of 2.0×10^6 . The commercial BMV marker was prepared according to Sambrook et al. (123) for all RNA gels.

In the second method, the following were combined: 25 ul of purified virus, 2 ul of 2-mercaptoethanol, 10 ul of formaldehyde, 10 ul of formamide and 1 mg of SDS. This mixture was incubated for 45 minutes at 65 C. Thirty-five microliters of each sample, approximately 26 ug of RNA, were loaded and then run at 40 V on a 1% agarose, formaldehyde denaturing gel according to Sambrook et al. (123). The gel was stained in either 1.5 ug/ml ethidium bromide in DEPC treated water or 0.5 ug/ml acrydine orange in DEPC treated water.

For the third method, approximately 21 ug of virion RNA was prepared by incubating purified virions for 1 hr at 37 C in dissociation buffer (0.1 M Tris, pH 8.0, 5% 2-B mercaptoethanol, and 4% sodium dodecyl sulfate) (57,58). Samples were prepared for electrophoresis in 1% LE agarose, 1/2x TBE (0.089 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA), 150V for 2 hr and stained afterward in 0.5 ug/ml of ethidium bromide in DEPC treated water. Standard markers were BMV and TMV RNA.

Viral coat protein molecular weight of purified virions was determined by 14% discontinuous SDS-polyacrylamide gel electrophoresis (58,85), 30 mA for 3 hr, and visualized by both Coomassie blue and silver staining (BioRad Labs, Richmond, CA). One hundred microliters of concentrated MT-RMV-V virions, approximately 53 ug, were solubilized in 4% SDS, 5% 2 B-mercaptoethanol, 20% glycerol, 0.1 M Tris, pH 8.0 and boiled 5 min. prior to loading. An 80 ul sample was loaded into each lane. Low molecular weight standards (BioRad) and TMV coat protein were used as markers. Purified NY-RMV was included as an additional marker and for comparison purposes.

Thin Section Electron Microscopy of Infected Plant Tissues

Coast Black oats were inoculated with MT-RMV-V by infective R. maidis at 14 to 16 days post sowing and allowed to feed as described earlier. At 21 to 28 days post

inoculation the outer leaf margins, of leaf sections showing slight chlorotic mottling, were harvested and prepared for examination with the Zeiss EM 10CA electron microscope.

Leaves were cut into approximately 1 mm² pieces and fixed overnight at 4 C in 3% glutaraldehyde in 0.2 M sodium potassium phosphate (NaK₂PO₄) pH 7.2. Post fixation was in 2% osmium tetroxide (OsO₄), followed by dehydration in a 70-100% ethanol series. Samples were treated with propylene oxide and then infiltrated with Spurr's epoxy resin. Ultrathin sections were cut with a diamond knife (DuPont, Wilmington, DE) on a Reichart OM-U2 ultramicrotome and doubled stained with uranyl acetate followed by Reynold's lead citrate. Samples were observed at 60 kV.

Polyclonal Antisera Production

Polyclonal antisera to the MT-RMV-V isolate of BYDV were raised using two, young adult New Zealand white rabbits. Fractions containing purified, intact virions, as per electron microscopic assay, were used as the antigen source. Initially, 1.0 cc of virus containing fractions, approximately 210 ug of virus per ml, were mixed with an equal amount of Freund's Complete adjuvant (Sigma). Subsequent booster injections using a 1:3 ratio of freshly prepared virus containing fractions and Freund's Incomplete adjuvant (Sigma) were made at 2 week intervals for 6 weeks. Blood was drawn during the intervening weeks.

Antisera were tested for specificity to the antigen by indirect ELISA (19,76) using clarified homogenates from virus infected leaves as test samples. The homogenates were produced by following the virus purification procedure through the resuspension of the PEG/NaCl precipitation step. One hundred microliters of homogenate sample were placed in each well in Immulon II ELISA microtiter plates (Dynatech, Rockville, MD). The homogenate was incubated for 3 hr at room temperature. After washing in PBS + 0.5% Tween-20, the wells were blocked with 0.2% egg albumin before the addition of 100 ul of each polyclonal antiserum individually to each well. Incubation of this step was for 4 hr at room temperature. Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase were used to detect positive samples.

Monoclonal Antibody Production

MT-RMV-V antigen was purified following the method of D'Arcy et al. (24). A 1:1 ratio of antigen, approximately 100 ug, and Complete Freund's adjuvant (Difco, Detroit), total volume equalling 0.5 cc, were injected intraperitoneally into each of 4 six week old, male BALB/c mice. Booster injections were given intravenously, in the tail, 6 weeks after mice were immunized.

A three week old, female BALB/c mouse was anesthetized and its thymus surgically removed. Thymocytes were washed and teased apart in a glucose sodium-phosphate buffer,

centrifuged at low speed and resuspended in HAT medium [Dulbecco's Modified Eagle Medium (GIBCO), 100 uM hypoxanthine, 0.4 uM aminopterin, 16 uM thymidine (Sigma) and 10% heat inactivated horse serum (Hyclone, Logan, UT)]. Feeder cells were plated at 2×10^5 cells/well and incubated in 24-well, flat bottomed culture plates (Corning, Corning, NY) at 37 C.

Three days after boosting immunized mice, spleens were aseptically removed from 2 mice and were used in separate fusions with P3-X63-Ag8.653 BALB/c plasmacytoma myeloma cells (American Type Culture Collection, Rockville, MD) (38,51,69,72,81,135). One milliliter of hybridoma culture was placed into each of 144 tissue culture wells containing 1 day old thymocyte feeder cells per fusion. Hybridomas were incubated at 37 C for three days without feeding. Every third day, 1/2 of the media was aspirated and fresh HAT media added. After 12 days HT media, HAT media without the aminopterin, replaced HAT media.

Hybridomas were initially screened by indirect ELISA for antibody specificity (see above for procedure). Goat anti-mouse antibodies conjugated to alkaline phosphatase, at a 1/500 dilution, were used to detect positive samples. Positive hybridoma wells were cloned by limiting dilution cloning and again screened by indirect ELISA for monoclonal antibody production. When screened against purified virus

preparations, 50 ul of each virus preparation was added per well, and 100 ul of each monoclonal supernatant was used.

Results

Virus Purification and Assay

Determination of average virus yield was based on data from 11 purifications. Spectrophotometric readings of purified virus preparations taken at A_{260} , A_{280} and A_{320} indicated that the virus yield ranged from 1.47 mg/kg to 7.21 mg/kg with a mean yield of 4.2 mg/kg of infected, plant starting material (S.D. = 2.04 mg/kg, n=11). The $A_{260/280}$ absorbance ratio was 1.84 (S.D. = 0.23, n=10) for the MT-RMV-V isolate based on spectrophotometric readings from 10 virus purifications.

MT-RMV-V virions were purified from all gradient fractions except F-1, although virions were observed in that fraction. In general, F-2 or F-3 fractions had the highest concentration of virus particles and were the cleanest preparations, as per electron microscopic assay. Most virions appeared isometric in shape, although some looked swollen or ruptured (Fig. 1.). An arrow in Fig. 1c points to a ruptured virion. Swollen virions are marked with an "*" in Fig. 1. A total of 559 virions were measured from 11 virus purifications. Virion diameters ranged from 23.0 nm to 27.0 nm with an average of 24.7 nm (S.D. = 1.2 nm).

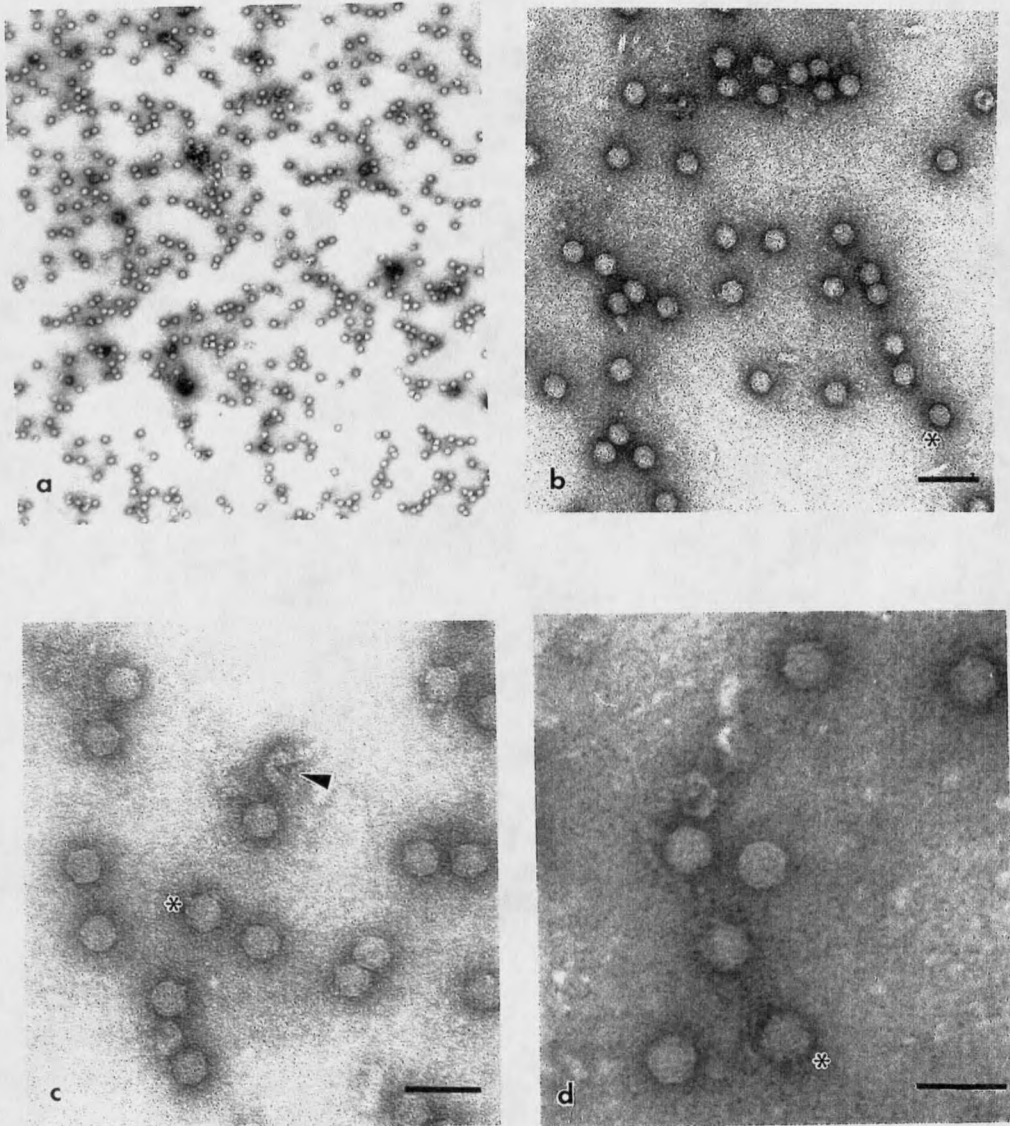


Fig. 1. Purified virus preparations of the MT-RMV-V isolate of BYDV. a) x 27,500 b) x 109,400 c) x 175,000 d) x 218,700. Bar represents 50 nm.

Estimation of Nucleic Acid Size

Electrophoresis of extracted viral RNA in formaldehyde denaturing gels was unsuccessful. No viral RNA bands could be detected. Using intact virions, dissociating the nucleic acid from the coat protein and then denaturing the viral RNA was slightly more successful. An extremely faint band was detected with an apparent Mr of 1.70×10^6 . This size correlates to approximately 5.1 Kb in length. A single RNA band was observed with an apparent Mr of 1.65×10^6 in the non-denaturing gel (Fig. 2.). This size would correlate to having approximately 4955 nucleotide bases in the virus genome.

Estimation of Coat Protein Size

A major polypeptide band was observed having an apparent Mr of 21.0 to 21.7 Kd (Fig. 3.). Occasionally, 1 or 2 smaller minor bands with apparent Mr's of 20.6 Kd and 18.7 Kd were seen when SDS-PAGE gels were stained with silver. A polypeptide doublet with apparent molecular weights of 59.0 Kd and 60.0 Kd were also seen.

Cytopathology of Infected Tissues

Sieve elements, phloem parenchyma and companion cells contained virions and/or displayed various cytopathological alterations induced by virus infection in the plant. Virions were detected in only a few types of cells. Frequently they were located in electron dense areas

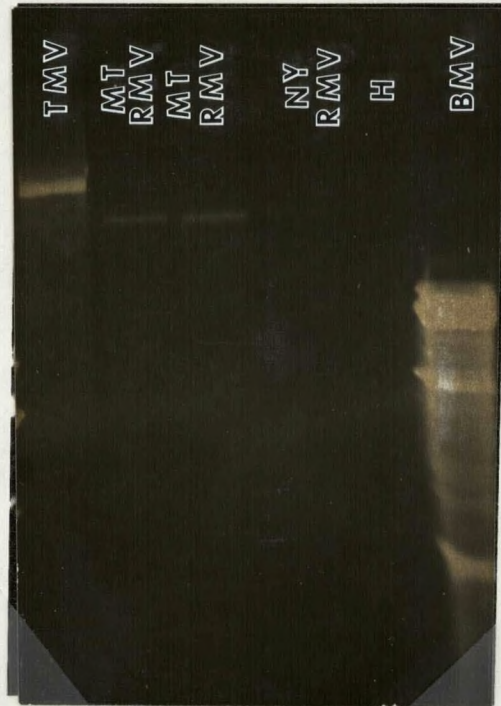


Fig. 2. Ethidium bromide staining of a non-denatured nucleic acid gel. Lane 1 contains TMV RNA; lane 2, MT-RMV-V; lane 3, MT-RMV-B; lane 4, NY-RMV; lane 5, healthy; and lane 6, BMV RNA.

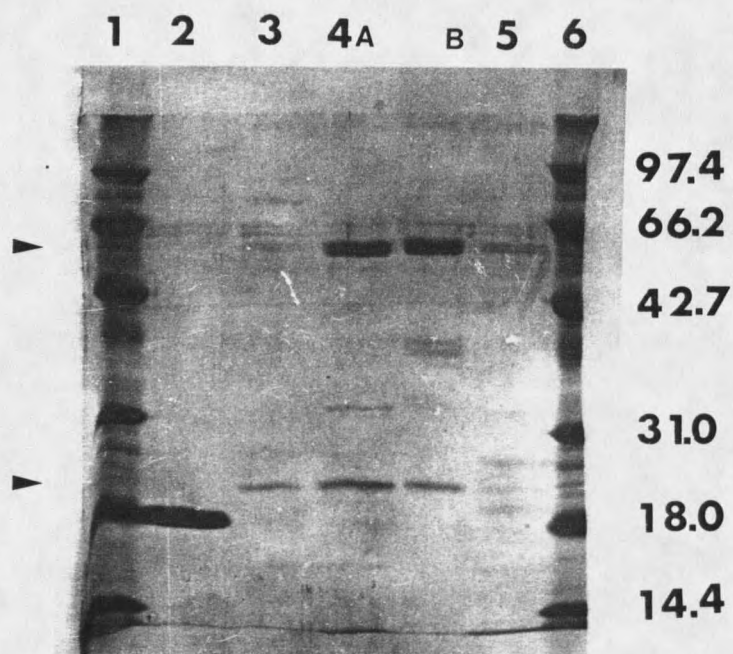


Fig. 3. Coomassie blue staining of a 14% SDS-PAGE protein gel. Lanes 1 and 6, BioRad low molecular weight markers; lane 2, TMV; lane 3, MT-RMV-V; lanes 4a and b, MT-RMV-B; lane 5, NY-RMV.

(arrows) (Fig. 4). Phloem cells adjacent to virus containing cells often were normal in appearance when compared to healthy cells (133) (Fig. 4). Virions were readily visible in the cytoplasm (Fig. 5 arrows). In addition, infected cells exhibited extensive deposits of what appears to be callose along the inner cell walls in MT-RMV-V infected material (Fig. 6). Electron dense areas of Fig. 6. contained highly aggregated clumps of virions. Increased numbers of irregularly shaped vesicles, with or without light staining fibrils or darker staining amorphous material, were also present in virus containing cells (Fig. 7). A light-staining matrix of fine fibrous material was seen throughout the background cytoplasm of diseased cells whether or not virions were present. Highly infected, disintegrating phloem cells containing numerous virions lacked intact organelles; however, those cells not containing virions generally had normal appearing mitochondria, proplastids and nuclei.

Cellular material similar morphologically to what has been identified as callose by other workers (45,47), occluded sieve plate pores and covered the inner cell walls of sieve tube elements. Figure 8 shows an enlarged view of a longitudinal section through a sieve plate between two cells showing cytopathological alterations. Note the remnants of heterochromatin which are indicated by arrows in the figure. No endoplasmic reticulum was found near the



Fig. 4. Longitudinal section through phloem cells of oats, *Avena byzantina* (Koch) cv. Coast Black infected with BYDV MT-RMV-V. The two cells on the left are infected, the two cells on the right are not. x 8,750. Mitochondria (M), proplastids (P).

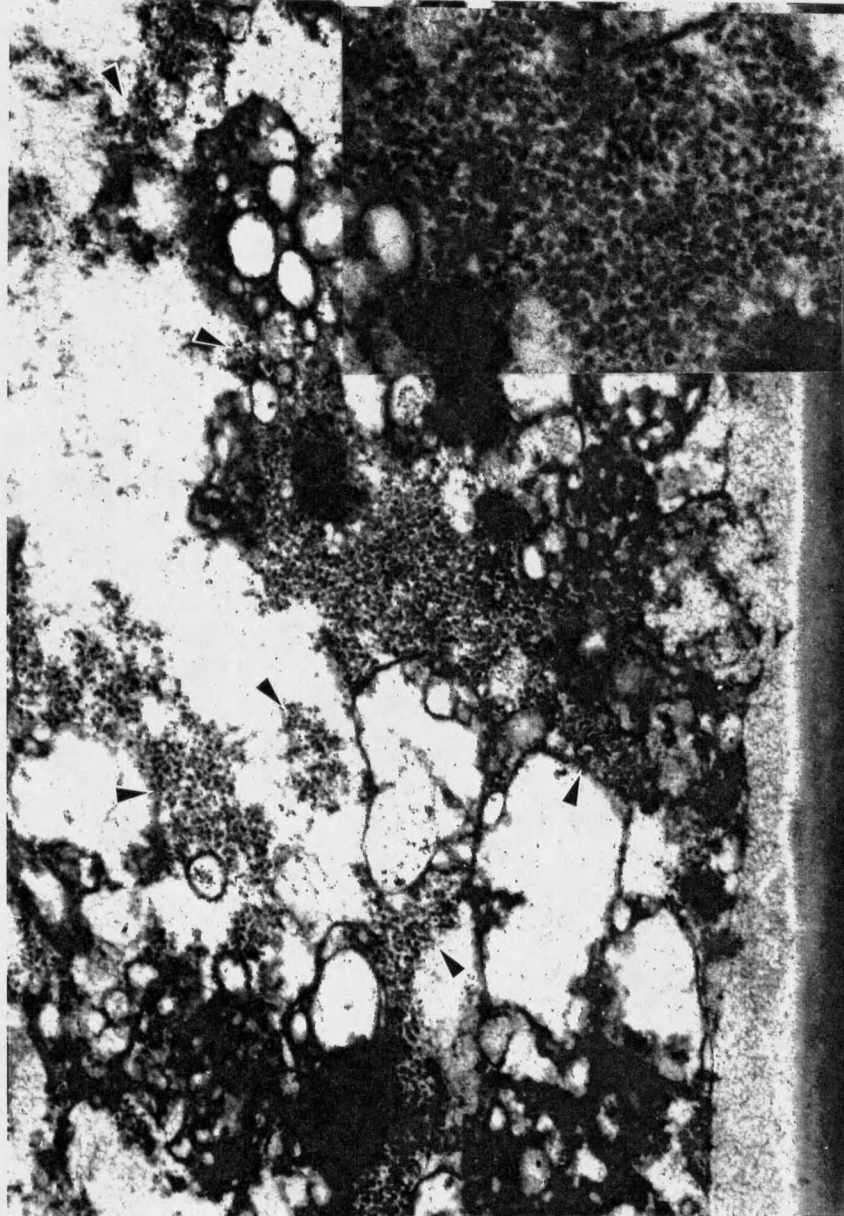


Fig. 5. Distinct clumps of MT-RMV-V virions in an infected oat cell. x 86,000. Note the absence of a crystalline arrangement of virions (inset). x 90,000.



Fig. 6. Part of an inner cell wall callose deposit (C).
x 17,500.

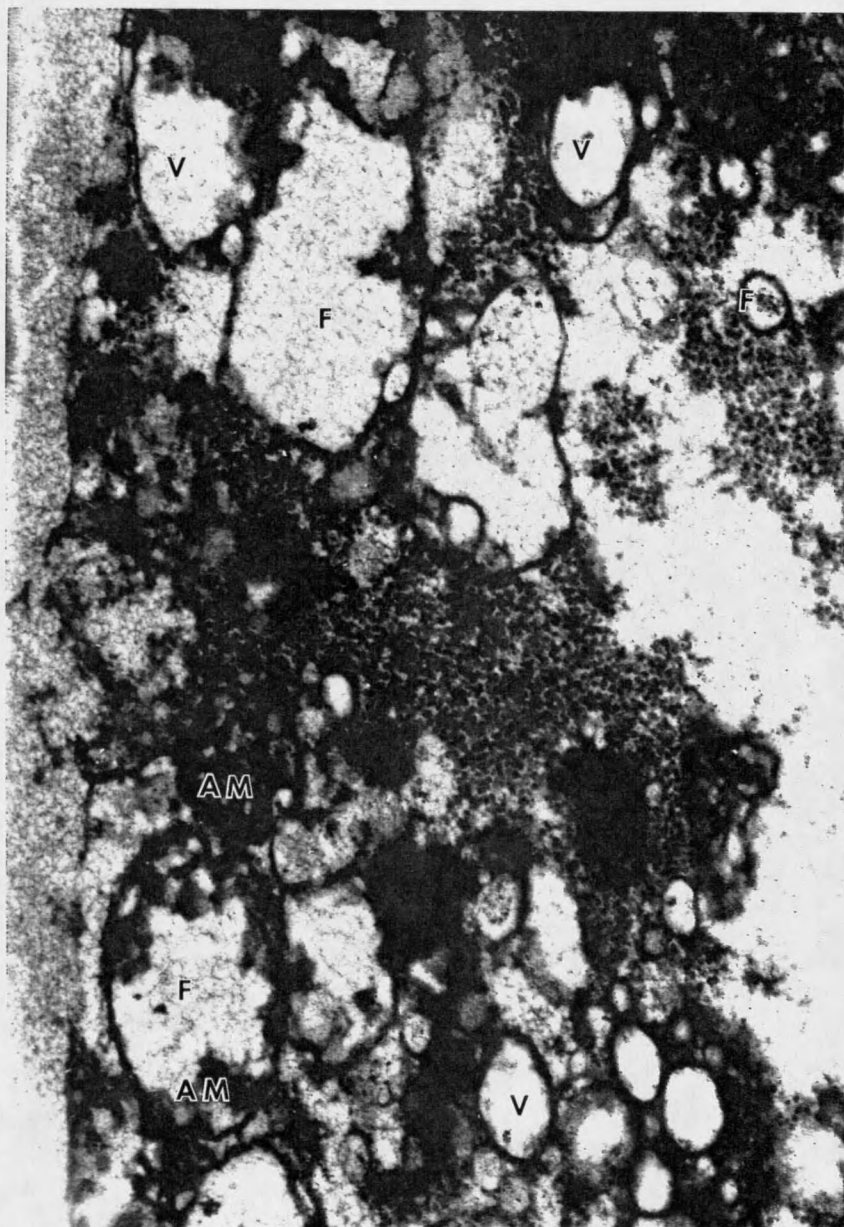


Fig. 7. Numerous irregularly shaped vesicles (V) in a portion of an infected oat phloem cell. Some vesicles contain fibrillar material (F) or dark staining amorphous material (AM). Background cytoplasm contains increased amounts of light staining fibrous material. x 87,500.

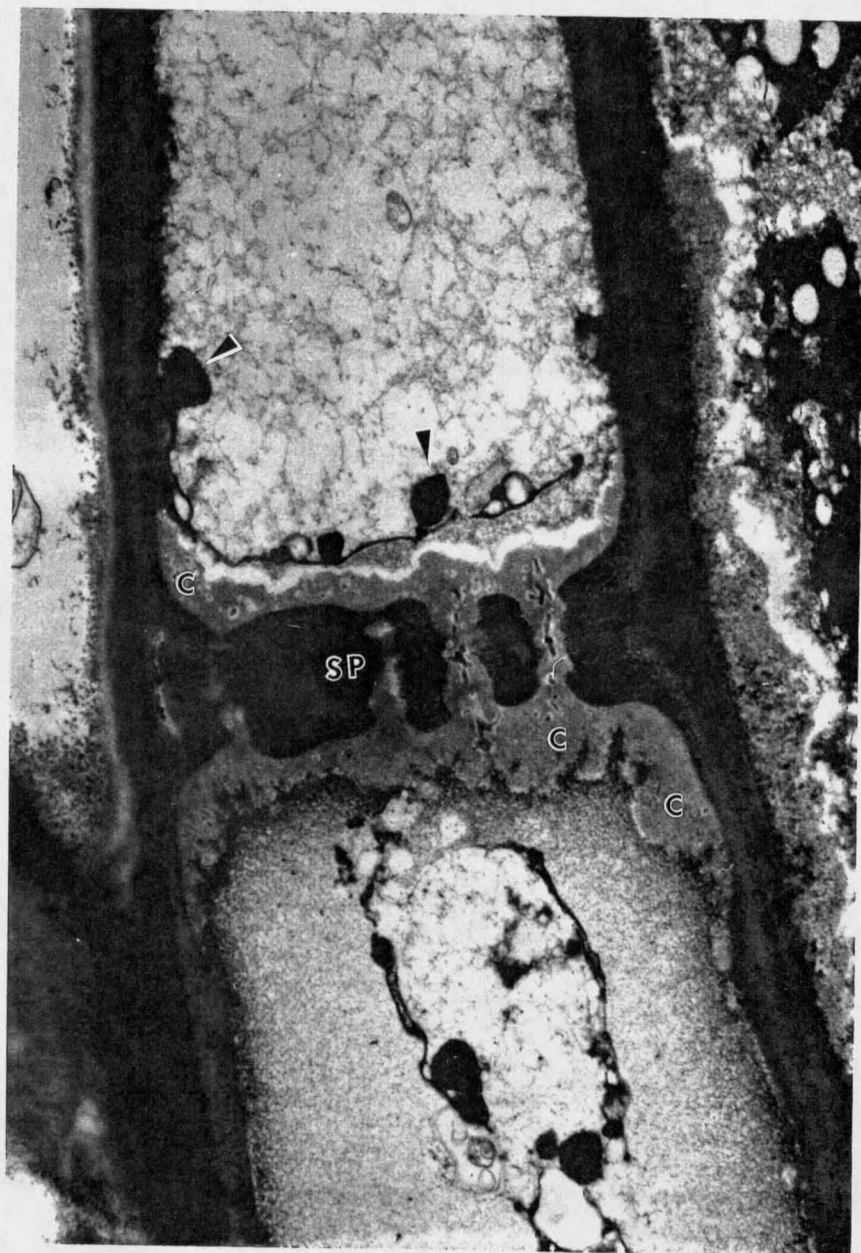


Fig. 8. Callose (C) occluded sieve plate pores (SP).
x 17,500.

occluded sieve plate pores. Two types of tubular membranes could be detected in the cytoplasm of some of the samples examined (Figs. 9 and 10). Both types were found in healthy oat samples, but appeared to be more prevalent in virus infected leaf material. One type was a rod like, branched tubule while the second type appeared flexible and did not have a consistent diameter. The branched tubules were not uniformly dispersed in the cytoplasm. They did appear to be continuous with other cell membranes as indicated by arrows in Figure 9. Highly proliferated, tubules of narrow diameter and small spherical particles with radial arms, were seen infrequently.

Preliminary Evaluation of Polyclonal Antisera

Both rabbit polyclonal antisera reacted more strongly with extracts from MT RMV-V infected tissues than with extracts from healthy leaves in preliminary testing by ELISA (Table 1). Antisera #899 produced a higher A_{410} reading than that of antisera #898 in the positive wells. Positive wells were considered those with readings of at least two times that of healthy readings. Both antisera were unable to distinguish between the MT and the NY isolates of RMV despite the use of the MT isolate as the inject antigen.

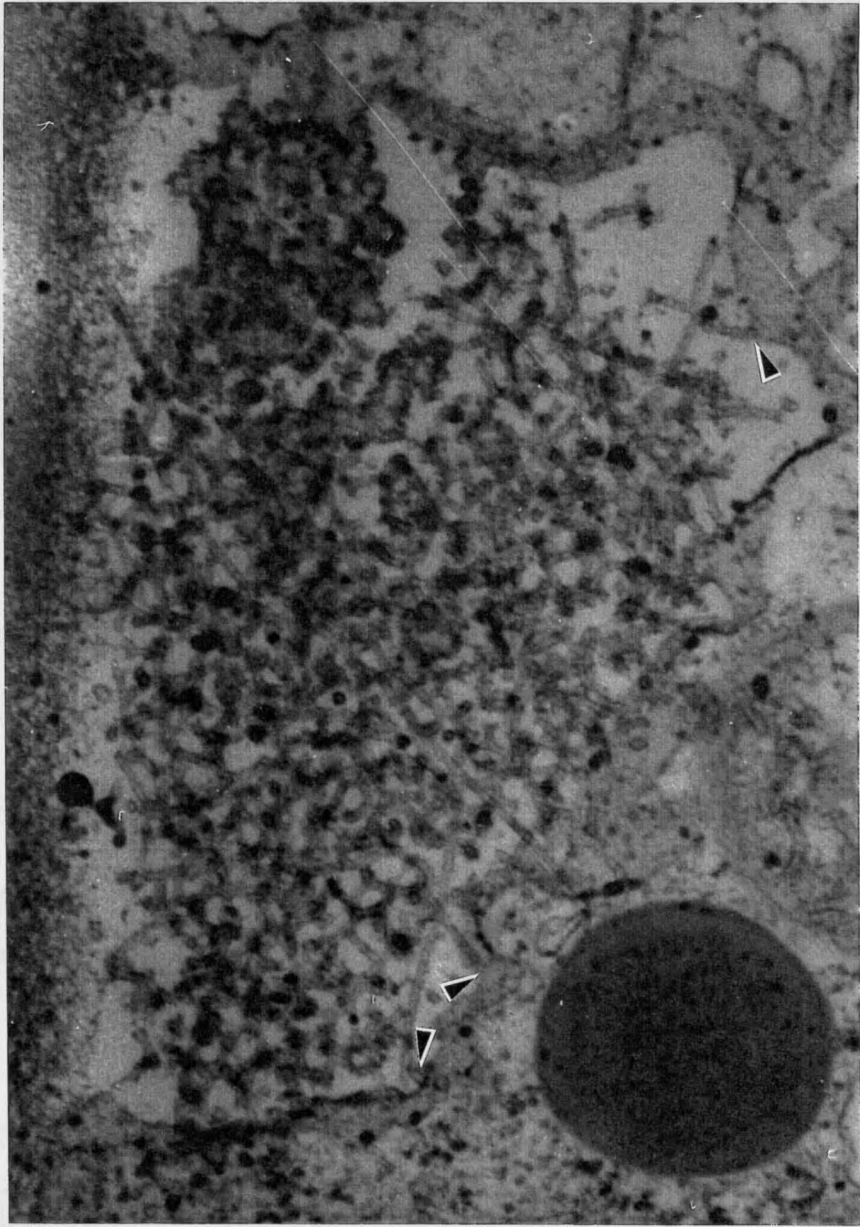


Fig. 9. Rod like tubular membranes found in phloem cell cytoplasm. x 87,500.

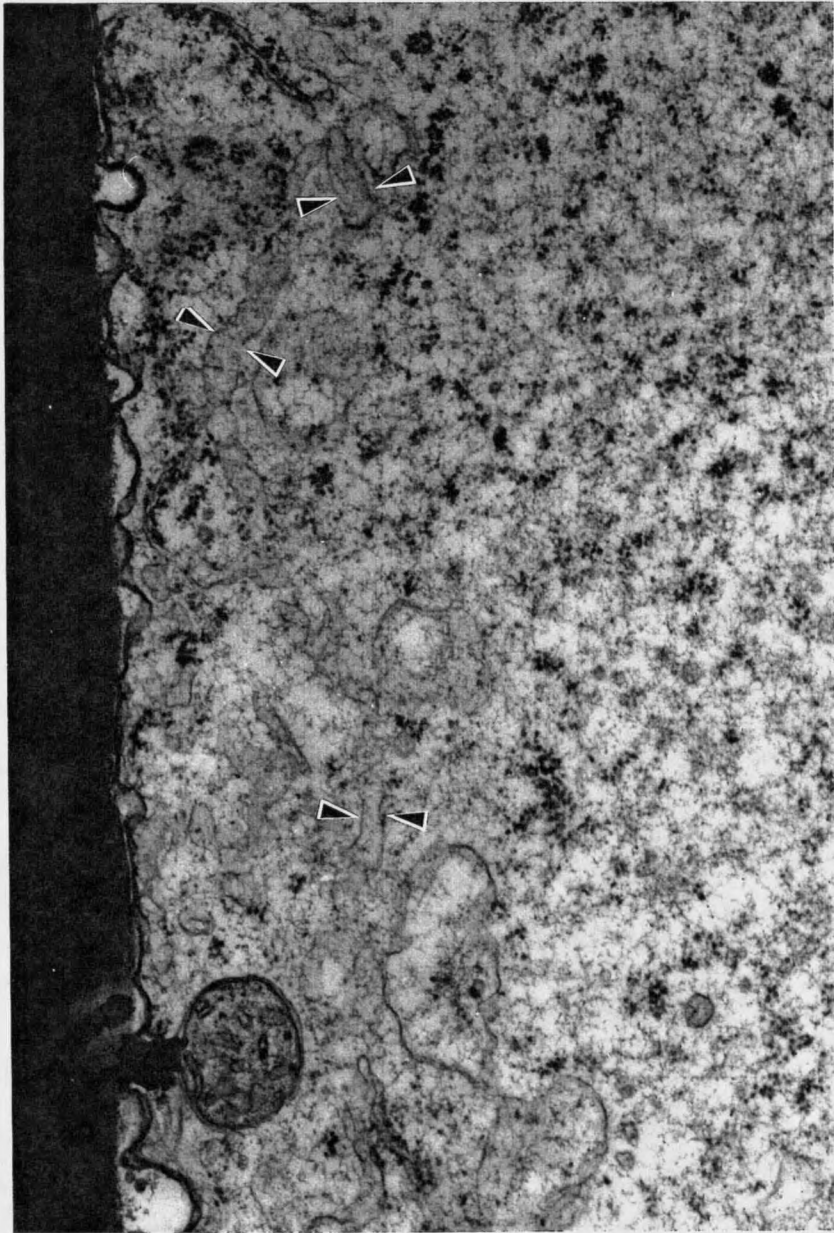


Fig. 10. Non-rigid tubules with varying diameters (arrows) found in phloem cell cytoplasm. x 27,500.

Table 1. Preliminary evaluation of two MT RMV-V polyclonal antisera by indirect enzyme-linked immunosorbent assay (ELISA) using clarified leaf homogenates from oats infected with MT RMV-V or NY-RMV or from healthy oats.

Antiserum	Absorbance at 410 nm		
	MT	NY	HEALTHY
#898	0.05	0.09	0.00
#899	0.12	0.12	0.03

(n=6 for each test sample)

Preliminary Evaluation of
Monoclonal Antibodies

Of the 288 mixed hybridoma cell cultures screened, 26 cell cultures were retained for limiting dilution cloning. Initially, six monoclonal antibodies were elicited against a preparation from RMV infected plant material in preliminary testing. Table 2 shows preliminary ELISA screening results of those monoclonal antibodies against four BYDV isolates and healthy plant sap using 100 ul of clarified leaf homogenates as test samples. Each monoclonal supernatant was screened against four replicates of each leaf homogenate to determine whether further testing was warranted. Table 3 shows ELISA screening results of two of these monoclonal lines, four additional monoclonal lines, and one mixed hybridoma cell line against purified MT-RMV and NY-RMV virions and healthy plant sap. This screening was used to

Table 2. Preliminary evaluation of six monoclonal antibodies from two separate fusions against MT RMV-V, NY-MAV, NY-RPV and NY-PAV infected and healthy plant sap by indirect enzyme-linked immunosorbent assay (ELISA).

Antibody	Absorbance at 410 nm				
	MT-RMV-V	NY-MAV	NY-RPV	NY-PAV	HEALTHY
A.1-B.5 (H2)	0.54	0.17	0.02	0.00	0.00
A.2-A.3 (D8)	0.16	0.10	0.00	0.00	0.00
A.5-A.1 (C11)	0.81	0.39	0.03	0.15	0.06
A.5-A.1 (E1)	1.23	0.57	0.21	0.38	0.17
B.1-B.1 (C8)	0.53	0.28	0.05	0.10	0.06
B.1-B.1 (E9)	0.48	0.18	0.04	0.00	0.02

(n=4 for each monoclonal line)

Table 3. Preliminary evaluation of antibodies from one mixed hybridoma cell line and six monoclonal cell lines against purified virions of Montana and New York RMV isolates of BYDV by indirect enzyme-linked immunosorbent assay (ELISA).

Antibody	Absorbance at 410 nm						
	Leaf Homogenates			Purified Virions or Control			
	MT-RMV-V	NY-RMV	HEALTHY	MT-V	MT-B	NY	HEALTHY
Hybridoma:							
A.2-A.3	0.16	0.23	0.08	-	-	-	-
Monoclonal:							
A.2-A.3 (1)	-	-	-	0.16	0.18	0.17	0.06
A.5-D.2 (1)	-	-	-	0.51	0.54	0.59	0.17
B.1-B.1 (1)	-	-	-	0.35	0.36	0.39	0.15
B.1-D.3 (1)	-	-	-	0.26	0.15	0.17	0.11
B.3-D.6 (1)	-	-	-	0.43	0.40	0.32	0.12
B.3-D.6 (2)	-	-	-	0.14	0.23	0.15	0.02

(n=6 for the hybridoma line; n=2 for the monoclonal lines)
(- = not tested)

determine the potential for a monoclonal antibody to distinguish between two serologically indistinct virus isolates. This screening was done twice for each monoclonal. There was relatively little difference between ELISA readings for the MT-RMV or NY-RMV BYDV isolates.

Discussion

Virions were obtained from all our MT-RMV-V purifications performed. This is significant since the consensus among BYDV workers has been that RMV isolates have been notoriously difficult to purify. The ultraviolet spectrophotometric readings indicate that although the virus yields varied widely, the average yield was comparable to those reported by D'Arcy et al. for a PAV isolate (24). This finding was not unexpected as D'Arcy et al. (24) also reported a wide range in virus yields. BYDV virus titer in plants can fluctuate, not only based on the time of year that infection occurs, but also on the post inoculation time at which the infected plants were harvested (5,20,21,35,75,96,128). In 1988, Webby and Lister (146) reported their success at purifying an RMV isolate. Their yields ranged from 2-400 ug/Kg of infected leaf material. Our purification procedure greatly increased our average MT-RMV-V yield (4.2 mg/Kg), compared to the procedure of Webby and Lister (146). The $A_{260/280}$ ratio, which not only is an indication of the purity of the virus preparation, but is

an indication of the chemical composition of the virions, for MT-RMV-V was comparable to those reported for bean leaf roll virus (BLRV) (17), beet mild yellowing virus (BMYV), a BWYV strain (26), and some of the other Luteovirus members (16,17,144). Still, individual readings also varied and perhaps indicate that some of the samples were not as clean as they should be.

The average diameter of the particles was similar to other BYDV isolates studied (16,17). In general, most Luteoviruses have diameters within the 24 nm to 27 nm range, and the MT-RMV-V isolate was no exception.

Electrophoresis of the MT-RMV-V nucleic acid was conducted using both denaturing and non-denaturing RNA agarose gels. Although giving only a rough estimate of nucleic acid size, running the non-denaturing gel was a satisfactory method to quickly determine the presence of detectable RNA in a particular sample. Ethidium bromide can stain non-denatured RNA more effectively than denatured RNA. In the course of denaturing the RNA samples, the RNA may have been nicked and may have produced a sample composed of many smaller fragments. No direct evidence of fragmentation of the RMV RNA samples has been produced. However, the results of subsequent Northern blot experiments indicate that fragmentation of the RNA may have occurred by the presence of a band beneath the putative full length RNA band. Whether or not fragmentation occurred, the

combination of an initially low concentration of RNA and the relative inefficiency of ethidium bromide and acridine orange to stain denatured RNA, may be the reason for the lack of a visible and distinct RNA band. Although the two nucleic acid measurements obtained were similar in size, this size was smaller by approximately 500-650 bases from the other Luteoviruses in which the complete nucleic acid sequences are known. Obviously, sequencing the entire viral genome would give a much more accurate sizing of the nucleic acid.

Estimating coat protein size for the MT isolate was relatively simple once an adequate purification procedure was developed for the RMV isolates. It was assumed that the polypeptide band of about 22 Kd from preparations of purified virions represented the coat protein. Western blot analyses were not done to positively identify the coat protein band, however. The apparent coat protein size was slightly smaller when compared to the coat protein sizes reported for NY-MAV, NY-PAV, NY-SGV and even for earlier reports of NY-RPV (16,17,124). Recently, a measurement of approximately 22.2 Kd was published for NY-RPV coat protein (141,142). This measurement is not much larger than the MT-RMV-V or the NY-RMV coat protein size reported here. Vincent et al. (142) also published revised coat protein sizes for many BYDV isolates based on nucleic acid sequences of the coat protein gene. In all cases, the true size of

the protein was smaller than the size calculated from the SDS-PAGE gels.

It is uncertain whether the 18.7 Kd or the 20.6 Kd protein bands, or either of them, represents the virus genome-linked protein (Vpg). Murphy et al. (83) reported a 17 Kd Vpg for an Illinois RPV isolate, whereas Vincent et al. (141) reported a size of 19 Kd for the NY-RPV isolate. The 59.0-60.0 Kd polypeptide doublet was similar in size to the 58 kd to 63 kd protein described by Vincent et al. (141) for the NY-RPV isolate. They speculated that this particular band, from NY-RPV, represents the readthrough coat protein gene, even though the size does not appear to correspond exactly to a full length readthrough protein. We did not attempt to further characterize the putative readthrough protein band from our MT isolate. The SDS-PAGE protein gels also showed that other polypeptides were present in the purified samples. This, of course, could be responsible for some of the variation in the calculated $A_{260/280}$ ratios.

The cytopathological examination of infected oat phloem cells generally corroborated earlier studies which distinguished RMV and RPV isolates by their cytological affects on plants (45). Gill and Chong (45) showed that a Manitoba isolate of RMV produced cytological alterations which were more similar in nature to those produced by the NY-RPV isolate than to either MAV or PAV isolates. These

similarities included the presence of vesicular membranous inclusion bodies that contained fibrillar material, and the presence of virus-like particles in distorted nuclei. The nuclear heterochromatin also showed progressive dissolution in both the Manitoba RMV and NY-RPV infected leaves (47). A fine fibrous matrix could be seen throughout the cytoplasm of both of these isolates. Except for the presence of virus-like particles in the nuclei, the cytopathology of the MT-RMV isolate resembled all of the above cytological alterations.

The differences in cytological alterations induced by the Manitoba RMV and the NY-RPV were many (45). In some instances, the MT-RMV isolate produced alterations similar to the Manitoba RMV isolate. Extensive callose, cell wall deposits and massive clumps of virions in the cytoplasm were unique to both of the Manitoba and MT RMV isolates, but not to the NY-RPV isolate. Plasmodesmata filled with densely staining material have been observed in RPV infected cells. This was not seen in the MT-RMV-V infected cells. Although dark staining masses of amorphous material could be seen in disintegrating MT-RMV-V infected cells as well as in RPV infected cells. No pathological alterations in mitochondrial shape or size in undifferentiated phloem parenchyma cells or companion cells were observed in the RMV infected plants; however, this was evident in RPV infected cells (45,47). Another major difference which distinguished

the effects between the Manitoba RMV isolate and RPV was the presence of highly proliferated tubular membranes and spherical particles with radial arms in the Manitoba RMV infected plants. These changes were not often seen in the MT-RMV-V infected cells we examined. This may be because our examination used slightly older, infected plants and a time course examination of virus infection and its effects was not performed. However, an overall increase in the presence of membranous tubules in infected plants was noticed when compared to healthy oats of the same age. Gill and Chong (47) reported the presence of non-dispersed branched tubules in RPV infected cells but not in Manitoba RMV infected cells. Branched tubules were found in our MT-RMV-V infected cells. This investigation shows that the cytological alterations caused by MT-RMV infection are not consistent with either Manitoba RMV or RPV induced alterations. In fact, the cytological alterations seem to reflect a variety of changes, thus making sub-group classification more challenging. Perhaps the MT-RMV-V isolate should be considered to fall somewhere within a continuum of cytological alterations, with those alterations induced by the Manitoba RMV and RPV at opposite ends. Developing additional sub-groups based on the alterations induced by the MT-RMV-V isolate is not warranted. Even though investigating cytological alterations of infected plants could be used as supporting evidence for classifying

a particular BYDV isolate, routine electron microscopic work was not considered time, labor or cost effective. Cytopathological properties are no longer considered a usable technique for the identification of BYDV isolates.

The polyclonal and monoclonal antibody experiments have shown that despite using the MT-RMV-V isolate as the injected antigen, the antibodies could not distinguish between the NY and MT RMV isolates. This was also the case when using NY-RMV derived polyclonals (15), however NY-RMV derived antibodies often gave weak reactions to some of the MT-RMV isolates. It is important, though, to have antisera or monoclonal antibodies that can detect different RMV isolates even though they may not distinguish amongst them. Based on previous work performed at Montana State University in the Spring of 1989 (data not included here), the mixed hybridoma cell line A.2-A.3 may have potential for use as a diagnostic for BYDV type isolates in general.

By modifying and developing a simple purification procedure for the MT-RMV-V isolate of BYDV, we were able to partially characterize this isolate. Its physical properties were very much like those of the other barley yellow dwarf viruses. Virion diameters, while not only similar to those reported earlier for BYDV isolates, indicated that the purification procedure does not apparently alter the particle size. Until the genome is completely sequenced, accurate values for RNA and coat

protein sizes cannot be determined. The MT-RMV-V isolate caused cytological alterations in diseased plants that were similar to those caused by a Manitoba RMV isolate and to those caused by RPV isolates. Unfortunately, the monoclonal and polyclonal antibodies were not successful in distinguishing between the MT and NY RMV isolates. This was not unexpected. Still, it is significant that the MT-RMV-V derived antibodies gave ELISA readings of similar strength for both MT and NY RMV isolates and that they can detect RMV isolates in general. The purification and characterization of the MT-RMV-V isolate is an important step in gaining more knowledge about the variety of BYDV isolates.

CHAPTER 3

MOLECULAR INVESTIGATION

Introduction

Taxonomic classification within the Luteovirus group remains in a state of flux. Since recognition of this plant virus group in 1975 (127), as few as three and as many as fifteen members, and a myriad of related strains and possible members, have been included in this group (17,78). Many of these viruses have not been studied within the last 10 to 15 years (17,26). Complete nucleotide sequences are known for only six isolates of three members: BWYV (140), PLRV (70,79,139), BYDV-PAV (80) and BYDV-RPV (142). A few partial sequences are known for soybean dwarf and carrot redleaf viruses (78,98,142,151).

Some workers have placed BYDV isolates into two subgroups based on their serological relationships (16,34,60,61,90,91,104,107,108,110,114) and cytopathological ultrastructure of infected phloem cells (45,47). Recently, dsRNA profiles (6,22,41,82) and to a limited extent, nucleic acid homologies (142) have helped delineate these groups. Group I consists of the MAV, PAV, and SGV isolates and Group II consists of the RPV and RMV isolates. Both groups

are serologically related in varying degrees to the other Luteoviruses (17,78). It has been suggested that BYDV Group II members be regarded as strains of beet western yellows virus (BWYV) (17,26,108), or as a separate cereal virus altogether (141). Vincent et al. (141) reported that the genomic organization and nucleic acid sequence of the BYDV-NY-RPV isolate is more closely aligned with BWYV and PLRV than to BYDV Group I members. However, differences in aphid vectors and host range have prevented them from definitively aligning NY-RPV with BWYV.

The other Group II member, RMV, has undergone little investigation because of previously inadequate purification procedures. Most studies have been limited to non-molecular investigations (13,15,45,46,149,150) and thus a definite taxonomic relationship based on molecular properties between BYDV Group I and II members and all Luteoviruses has not been made.

The use of polymerase chain reaction (PCR) methodology and restriction enzyme digest analysis to distinguish between selected Luteovirus members was reported recently by Robertson et al. (100). BWYV, PLRV, and the five NY type isolates of BYDV were readily detected and distinguished from one another using infected leaf material as the virus source. Although this was a breakthrough in Luteovirus diagnosis in terms of quick and unambiguous results, each of the viruses tested could be distinguished by other means,

e.g. time and labor consuming serology and aphid transmission studies. PCR methodology has an added benefit in that it can be used to help characterize individual isolates at the molecular level by enabling researchers to sequence specific fragments of the viral genome (7,37,88). It is no longer necessary to screen and sequence a random cDNA library of the virus genome for BYDV isolates.

Five Montana BYDV isolates collected in 1986 were initially identified as RMV-like on the basis of vector specificity. They were readily transmitted by Rhopalosiphum maidis (Fitch) (corn leaf aphid). Upon further investigation, it was determined that, although the MT RMV-like isolates were similar to the NY-RMV isolate serologically, the MT isolates appeared to be more virulent in Avena byzantina (Koch) cv. Coast Black oats than either NY-RMV or NY-SGV (15). Later it was shown that the MT isolates were also transmitted by Schizaphis graminum (Rondani) (greenbug). These findings seemed to imply that the MT-RMV isolates differed from the NY-RMV isolate of BYDV.

The object of these experiments, therefore, was to examine a MT RMV-like isolate at the molecular level. One goal was to distinguish between the NY-RMV isolate and a MT RMV-like isolate at the molecular level. Our second goal was to partially sequence the MT-RMV and NY-RMV genomes in order to gain basic knowledge of the molecular similarities and differences between them and the other Luteoviruses.

Materials and Methods

Nucleic Acid Isolation

Viral RNA was extracted using the proteinase K (Promega) and phenol method from purified MT-RMV-V and NY-RMV isolates as previously described in Chapter 1. Crude nucleic acids from 0.5 gm of BYDV infected oats (Avena byzantina cv. Coast Black) and BWYV infected sugar beets (Beta vulgaris) were obtained using the common phenol extraction method described by Robertson et al. (100) and Sambrook et al. (123).

DNA Primers

DNA primers were made using an Applied Biosystems ABI 381 (Foster City, CA) oligonucleotide synthesizer. Because the nucleic acid sequences of the MT and NY RMV isolates are not known, the primers used in this study were derived from published sequence data of BWYV, PLRV, and an Australian BYDV-PAV isolate (100). Primer Lu4 (5'-GTCTACCTATTTGG-3') pairs with bases 3453-3468 of an Australian BYDV-PAV isolate (80), bases 4084-4097 of BWYV (140) and bases 4207-4220 of PLRV (139). Lu1 (5'-CCAGTGGTTRTGGTC-3') is a two-fold degenerate primer which pairs upstream of Lu4 with first strand cDNA. Lu1 primer corresponds to the following bases: 2938-2952 of BYDV-PAV, 3564-3578 of BWYV and 3687-3701 of PLRV. Pol1 (5'-CGACTGCAGGGNTTYGAYTGG-3') is a 16-fold degenerate primer which contains a Pst I restriction site at its 5' end. It also base pairs to first strand cDNA and

corresponds to bases 2684-2695 of BWYV. Pol2 (5'-GCTGGATCCTGNSWRCARAAAYTC-3') is a 23-mer, 128-fold degenerate primer which contains a Bam HI restriction site. It pairs with bases 3041-3053 of BWYV. Fig. 11 is a simplified schematic showing the genomic locations where the DNA primers normally base pair. The Lu1 and Lu4 primers can hybridize with both viruses at the locations indicated. The Pol1 and Pol2 primers cannot hybridize with the PAV isolate. Each outlined box represents an open reading frame (ORF). As can be seen, overlapping reading frames are common. The BYDV NY-RPV isolate has the same genomic organization as BWYV (142). Translation products have been associated with most of the ORF's (78).

cDNA Production

A 10 ul sample of viral RNA or crude nucleic acid extract was combined with 1.5 ul water and 1 ul of 10 pM Lu4 or random primer (Promega) as reported by Robertson et al. (100). When viral RNA was used as the source template, 1 ul of 0.1 M MgCl₂ was added. Total volume remained at 12.5 ul. The cDNA was synthesized using an equal volume of reaction mix containing 0.5 units of the Superscript (BRL) moloney murine leukemia virus, RNase H- reverse transcriptase and incubated for 1 hr at 37 C. Sample volumes were increased to 50 ul with water and boiled to denature the reverse transcriptase.

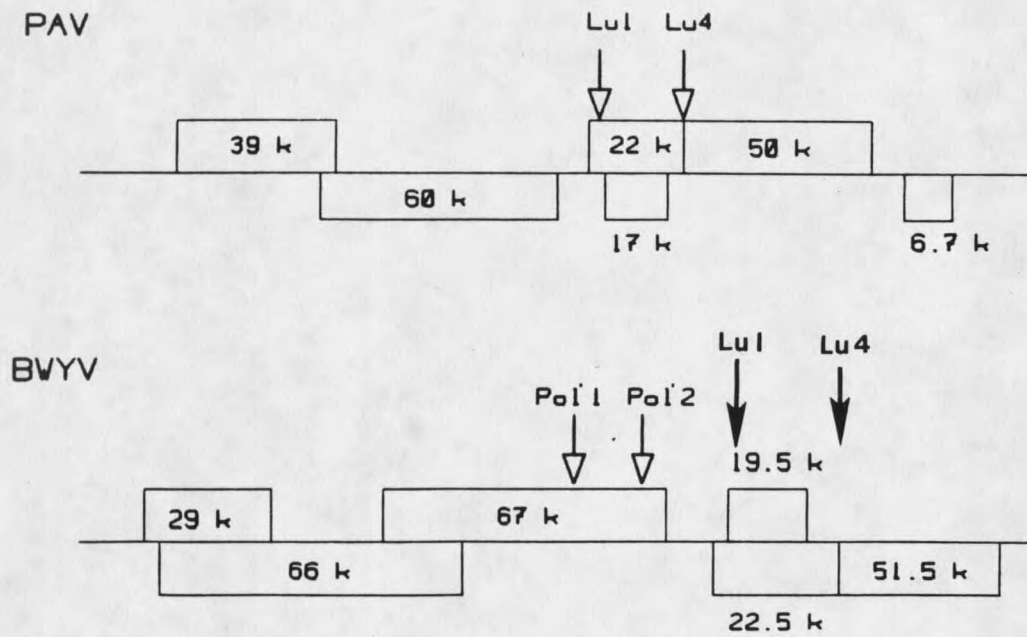


Fig. 11. Primer base pairing locations on a BYDV Australian PAV isolate and BWYV.

PCR

Specific virus encoded DNA fragments were amplified by the polymerase chain reaction method using Taq DNA polymerase (Promega) (53,122). Primer combinations were Lu1 - Lu4, Pol1 - Pol2 and Pol1 - Lu4. Reaction volumes of 100 ul were used. Only 5 ul of cDNA and 1 ul of 5 pM of each respective primer were needed. The temperature cycling regime was 1 min. at 94 C, 1 min. at 41 C, 3 min. to reach 72 C and 2 min. at 72 C for 40 cycles. All other temperature regimes were as according to Frohman (37), Robertson et al. (100) and Wittwer and Garling (147).

PCR Product Analysis

PCR products were visualized electrophoretically by loading 10 ul of product onto a 1.25% LE agarose gel (SeaPlaque, FMC Bio Products), running the gel at 150V in 1/2x TBE (0.089 M Tris-borate, 0.89 M boric acid, 0.002M EDTA) and staining with 0.5 ug/ml ethidium bromide in DEPC treated water (122). The 1.0 kbp DNA ladder (BRL, Gaithersburg, MD) and the 123 bp DNA ladder (BRL) were used as standards.

Restriction enzyme digests of the Lu1 - Lu4 fragment with Sau 3AI, Hae III, Hind III, Hinf I, Eco RI and Alu I (Promega and BRL) were made in order to differentiate between the MT-RMV-V, NY-RMV, NY-SGV isolates of BYDV and a CA isolate of BWYV. Three units of enzyme per 5 ul of PCR

product were incubated for 2 hr at 37 C and then visualized on a continuous, 10% polyacrylamide gel in 1x TBE with ethidium bromide staining. Alu I cut pUC 19 and Sau 3AI cut pUC 19 plasmids were used as markers.

Hybridization Analysis

A radioactive probe derived from the MT-RMV-V Lu1-Lu4 fragment was produced by reamplifying the fragment using PCR with 50 uCi [α 32 P]dCTP (DuPont) added (92,100,125).

Northern blots were made following the Zeta-Probe standard protocol (BioRad) using NY-RMV viral RNA and viral RNA from two MT-RMV-V isolates which were run on a non-denaturing gel. Southern blots using the Lu1-Lu4 PCR products of two MT-RMV-V and one other MT-RMV isolate, the 5 NY isolates of BYDV and CA-BWYV were done following the Zeta-Probe standard protocol, using low stringency conditions. Overnight blotting was in 40 mM NaOH followed by a rinse in 2x SSC (0.3 M NaCl, 0.003 M trisodium citrate). Pre-hybridization was in 1 mM EDTA, 0.5 M sodium phosphate (NaH_2PO_4), pH 7.2, 7% SDS at 65 C for 3.5 hr. Hybridization occurred overnight using fresh pre-hybridization buffer at 65 C. Prior to autoradiography, blotted membranes were washed twice with 1 mM EDTA, 40 mM NaHPO_4 , pH 7.2, 5% SDS then washed twice with 1 mM EDTA, 40 mM NaHPO_4 , pH 7.2, 1% SDS.

DNA Cloning and Sequencing

MT-RMV-V PCR fragments derived from primer sets Lu1-Lu4 and Pol1-Pol2 and NY-RMV PCR fragments derived from primer set Lu1-Lu4 were ligated into pUC 19 or pUC 119 plasmid vectors between the Pst I and Bam HI enzyme restriction sites of the multiple cloning region. Prior to ligation, fragments using Lu1 or Lu4 primers were reamplified using similar primers which also had internal Pst I or Bam HI sites. Plasmids were cloned into DH5 α F' E. coli (BRL). Minilystate plasmid preparations were made following the alkaline lysis method (123). Plasmids were purified from contaminants with the Geneclean II kit from Bio 101 (La Jolla, CA). Double stranded DNA inserts were sequenced by the dideoxy chain termination method using both universal and reverse primers with the Sequenase version 2.0 kit and protocol (United States Biochemical). Sequence retrieval and analysis were performed by the GenBank FASTA program (95) and ALIGN software (Sci. and Educ. Software, Silver Spring, MD), respectively. Enzyme restriction site mapping of the MT and NY Lu1-Lu4 PCR fragments were done by searching each sequence by computer. The sites were those recognized by Sau 3A1, Hae III, Hind III, Hinf I, Eco RI and Alu I restriction enzymes.

Results

PCR Product Analysis

Distinct bands representing specific PCR fragments were seen if the DNA primer sets were capable of base pairing with the individual Luteovirus examined (Figs. 12, 13, and 14). A major band of about 520 bp, as expected, was detected from all the Luteovirus infected leaf material and from each purified virus preparation tested when the Lu1-Lu4 primer set was used (Fig. 12). When the Pol1-Pol2 primer set was used, the expected 370 bp fragment was detected from MT-RMV-V, NY-RMV, NY-RPV and CA-BWYV infected material (Fig. 13). No corresponding fragment was detected from NY-PAV, NY-MAV or NY-SGV infected material, although other fragments were amplified from those samples. These fragments were not investigated. When the Pol1-Lu4 primer set combination was used, the expected circa 1.4 kb PCR fragment was detected in both the MT-RMV-V and NY-RMV samples (Fig. 14). No corresponding PCR fragments of 520 bp, 370 bp or 1.4 kb were ever detected from healthy Coast Black oats.

Enzyme restriction digests of the Lu1-Lu4 PCR fragments produced apparently unique banding patterns for each virus/isolate and for each enzyme tested (Fig. 15). In just one case, using Alu I, a similar sized band was detected from all four virus/isolate samples assayed, but the rest of the patterns were not the same. As expected, the digest

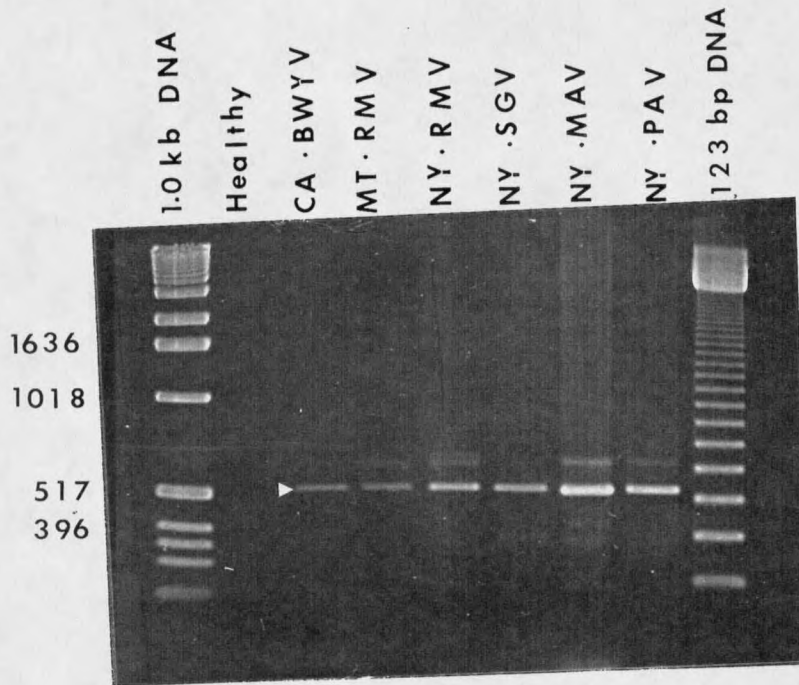


Fig. 12. PCR products using the Lu1-Lu4 oligonucleotide primers.

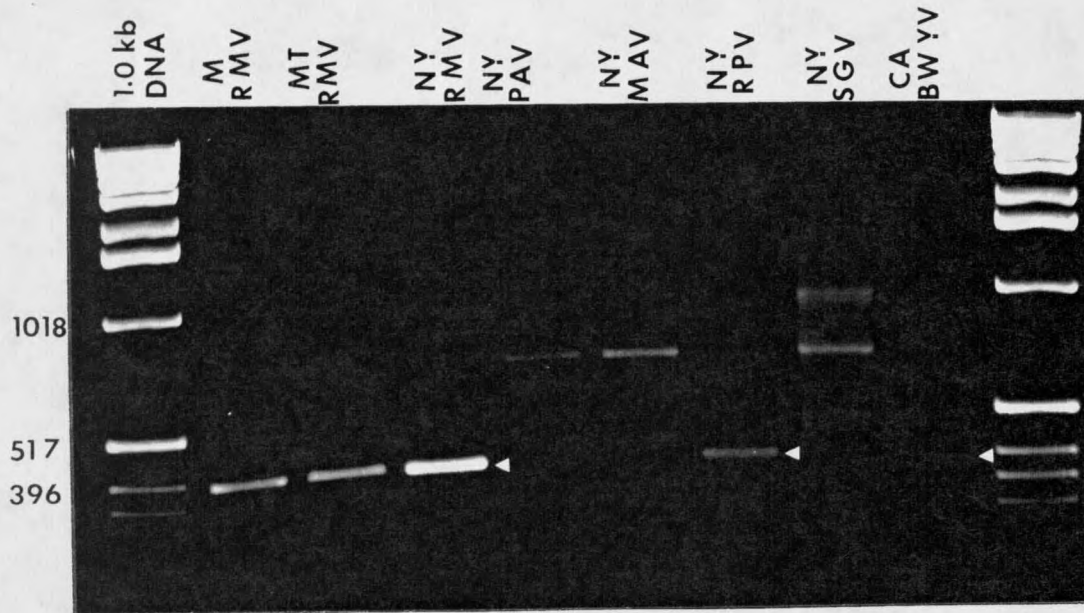


Fig. 13. PCR products using the Pol1-Pol2 oligonucleotide primers.

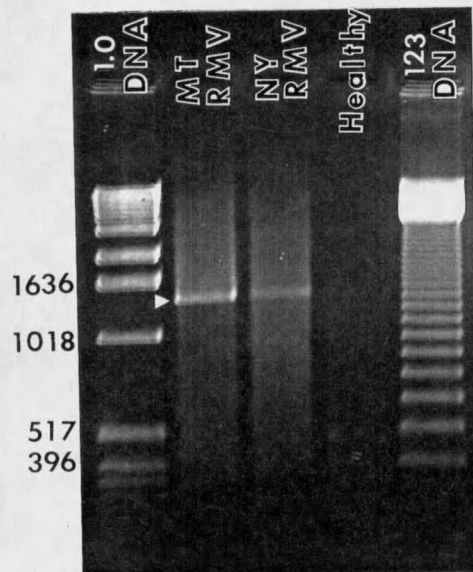


Fig. 14. PCR products using the Pol1-Lu4 oligonucleotide primers.

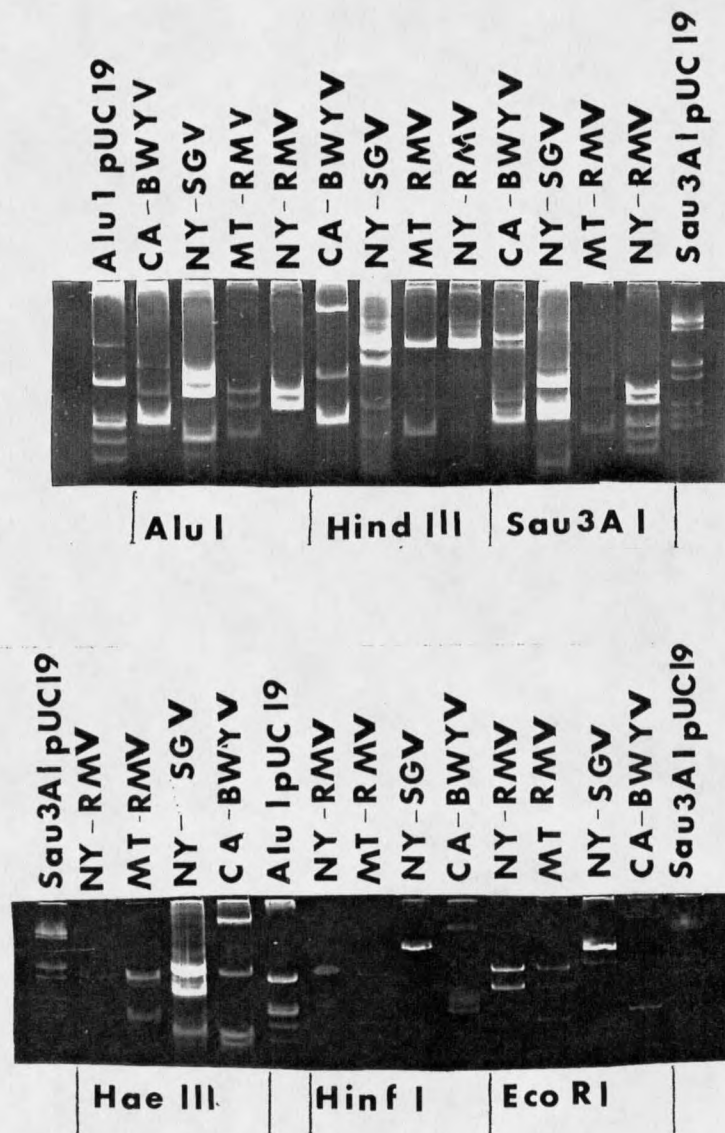


Fig. 15. Restriction enzyme digests of Lu1-Lu4 PCR fragments.

patterns were often similar between the MT-RMV-V and NY-RMV PCR fragments. However the two isolates could be readily distinguished on the basis of individual band size or the presence or absence of an additional band or bands.

Hybridization Analysis

A major band was observed on the autoradiographic film from the Northern blot in the lanes containing viral RNA from two separate MT-RMV-V purifications. A much lighter band, which had a similar migration to the MT-RMV-V RNA, was also detected in the NY-RMV lane (Fig. 16). A smaller band was also present in the sample lanes. Results from the Southern blot (Fig. 17) indicate that the radioactive probe was not as specific, since many bands were observed in the different sample lanes. This may be the result of low stringency blotting conditions. However a major band, of about 520 bp (arrow), was detected in the MT-RMV-V lanes, the MT-RMV-B lane, and the NY-RMV lane. No similar band was observed in lanes containing the other BYDV or healthy plant derived PCR products. Both autoradiographic films were exposed for one and three days.

DNA Cloning and Sequencing

Three individual clones carrying separate MT-RMV-V derived Lu1-Lu4 PCR DNA inserts and five individual clones carrying separate MT-RMV-V derived Pol1-Pol2 PCR DNA inserts were sequenced in both directions (Figs. 18 and 20). The

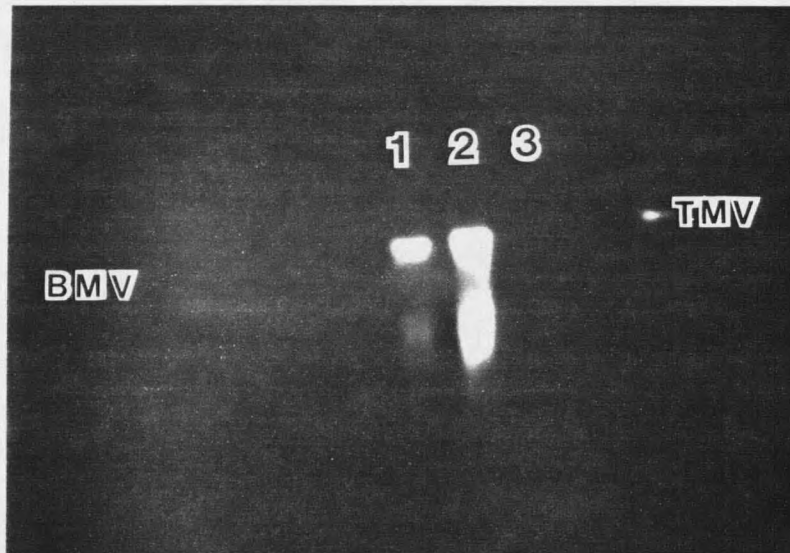


Fig. 16. Autoradiogram showing results of a Northern blot. Lane 1, MT-RMV-V; lane 2, MT-RMV-V; and lane 3, NY-RMV.

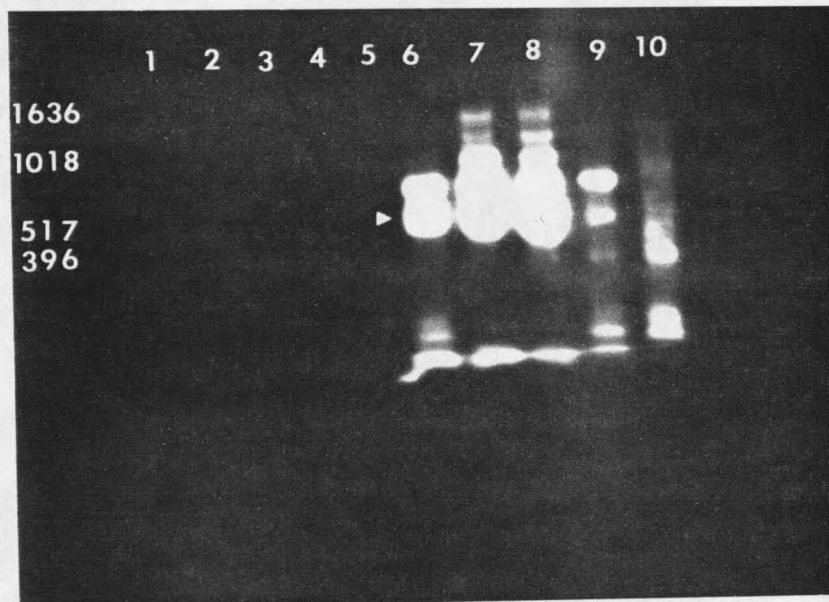


Fig. 17. Autoradiogram showing the results of a Southern blot. Lane 1, 1.0 kb DNA ladder; lane 2, NY-PAV; lane 3, NY-MAV; lane 4, NY-RPV; lane 5, NY-SGV; lane 6, MT-RMV-B; lane 7, MT-RMV-V; lane 8, MT-RMV-V; lane 9, NY-RMV; lane 10, healthy.

Lu1

1 GTGGTTGTGGTCGCGCAAACCCAGCGTAGACGCACCCGAAGACGAGGACGAC 52
 53 CAAGTGGAGACACTTCAGGAGGACCTCGAGGGCGAGGAGGCTCCGGGGAGAC 104
 105 TTTCGTATTTTCGAAGGACTCTATCGCGGGCAGTGCTCCGGAAAGCTCACCT 156
 157 TCGGGGCGTCTCTTTCTGAGTGCGCCAGCATTCTCTGGTGAATTCTCAAGG 208
 209 CCTACCATGAGTATAAGATCACAAAATCATACTGGAGTTCATCTCGGAGGC 260
 261 CTCTTCAACGCAGTCCGGTTCATCGCTTATGAGCTGGATCCCCACAACAAG 312
 313 CTCAGCACCCCTCGCATCAACAATCAATCAAATCTCGATCGTCAAGGGTGGCA 364
 365 AGCGSTCCATATCGTCCAAACAAATCGGAGGTGGAGTATGGCGAGATTTCGTC 416
 417 CGAAGACCAATTTGCAATACTCTACAAGGGCAGTGGAAACTCCTCAGTTGCC 468
 469 GGCTCGTTCGCGATCACGATGGTTCATACCCAAAATCCCAAATAGGTAGAC 520

Lu4

Fig. 18. Nucleotide sequence of the Lu1-Lu4 PCR fragment of MT-RMV-V. The positions of the Lu1 and Lu4 primers are indicated by underlining.

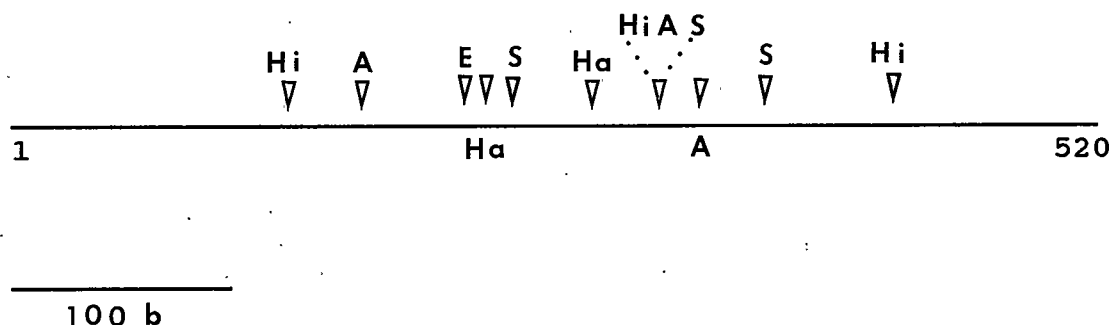


Fig. 19. Enzyme restriction map of the Lu1-Lu4 PCR fragment of MT-RMV-V. Hi=Hinf I, A=Alu I, E=Eco RI, Ha=Hae III, S=Sau 3AI.

underlined portions of each sequence are primer derived. There were no differences in nucleotide sequences obtained within each set of sequenced clones that could be attributed to the PCR process. Figure 19 shows the enzyme restriction sites in the MT-RMV-V Lu1-Lu4 sequence. Restriction sites were present for all enzymes tested except Hind III. Three individual clones with separate NY-RMV derived Lu1-Lu4 PCR DNA inserts were also sequenced in both directions and the enzyme restriction sites determined (Fig. 21 and 22). The nucleotide sequence obtained was the same for each of the three clones. Enzyme restriction sites were present for all enzymes tested except Hind III.

Pol1

1 TTTGATTGGAGTGTTGCGGATTGGATGCTCCAGGATGATAGGAAGGTCCGCA 52
 53 ACCGACTCACCCGCAACNNCAACACAGCTACACCAAGCGCTTCTTGCTGGTT 104
 105 GAAATGCATATCCAATTCTGTGTTGTGCTTGAGTGATGGCACTCTGCTAGCC 156
 157 NNAGCGCATNNNAGGAATCAGAAGTCTGGTTCTTACAACACCAGTTCGAGCA 208
 209 ACTCTCGAATCCGAGTGATGGNNNGCTATCATTGCGGTGCTACCTGGGCGAT 260
 261 GGCTATGGGTGATGATGCACTCGAGTCCAGTCGACACGAACTAGACGTGTAT 312
 313 AAAATACTAGGATTCAAAGTCGAGGTCTCAAACAACTGGAATTTTGTTC 364

Pol2

Fig. 20. Nucleotide sequence of the MT-RMV-V Pol1-Pol2 PCR fragment. The positions of the Pol1 and Pol2 primers are indicated by underlining.

Lu1

1 GTGGTTGTGGTCGCGGCTGGTCAGCGTCGACGCCGCCCCAGAAGACGAGGAC 52
 53 GACGAACTGGAAACACTTCAGGAGGATCTGGAGTCCGAAGAGGGTCGCGGGA 104
 105 AACATTTGTGTTTTTCGAAGGACTCTCTCACGGGCAATGCTCCGGGAAGCTCA 156
 157 CCTTCGGGGCGTCTCTATCAGAGTGCGCACGCATTCAGTAGTGAATTCTCA 208
 209 AGGCCTACCATGAATATAAGATCTCAAAGGTCACCTTGGAGTTCATCTCGGA 260
 261 GGCCTCTTCCAATCTGAAGGCTCCATCGCTTATGAGCTTGACCCACACAAC 312
 313 AAGCTCTCAGCTCTCTCTTCCACCATCAACGAGTTTTCAATCGTCAAAGGTG 364
 365 GTAAGAAGACCTTATCGTCCAATCAAATCGGAGGTGGAGTTTGGCGAGACTC 416
 417 AACAGAAGATCAATTCGCCATCCTCTACAAGGGTAATGGAAAATCCTCGATC 468
 469 GCGGGCTCGTTCCGCGTTACGATGGATGTTCTAACCCAAAACCCCAAATAGG 520
 521 TAGAC Lu4 525

Lu4

Fig. 21. Nucleotide sequence of the Lu1-Lu4 PCR fragment of NY-RMV. The positions of the Lu1 and Lu4 primers are indicated by underlining.

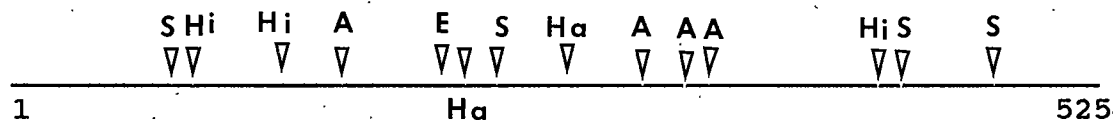


Fig. 22. Enzyme restriction map of the Lu1-Lu4 PCR fragment of NY-RMV. Hi=Hinf I, A=Alu I, E=Eco RI, Ha=Hae III, S=Sau 3A1.

The MT-RMV-V Lu1-Lu4 fragment shows 67%, 64.9%, 66.1% and 57.1% nucleotide sequence homology, in the coat protein region, with BWYV, PLRV, NY-RPV and Aus-PAV respectively. The NY-RMV Lu1-Lu4 fragment shows 66.2%, 65.2%, 64.1% and 57.1% nucleotide sequence homology, in the coat protein region, with BWYV, PLRV, NY-RPV and AUS-PAV, respectively. A 64.4% homology was noted between NY-RMV and bean leaf roll virus. There was 79.8% nucleotide sequence homology between the MT-RMV-V and NY-RMV isolates for this region. The deduced amino acid sequence of both the MT-RMV-V and the NY-RMV Lu1-Lu4 fragments were quite similar in many of the conserved areas to some of the other known Luteoviruses (Fig. 23). In areas of the gene where they differed from the rest, the MT and NY RMV sequences were similar to each other. Overall there was 77.1% amino acid homology between the MT and NY RMV isolates.

The small MT-RMV-V Pol1-Pol2 fragment shows 70% and 60% nucleotide sequence homology in the RNA dependent RNA polymerase region (ORF 2) with BWYV and PLRV, respectively. There was virtually no homology with the Aus-PAV isolate. The amino acid sequence of the MT-RMV-V Pol1-Pol2 fragment was nearly identical to BWYV, PLRV and NY-RPV (Fig. 24).

MT-RMV	1	VVVVAQT	QRRRTRRRGRPSGD	TS	GGPRG	RGGS	G	ETFVFS	K	40
NY-RMV	1	VVVVAAG	QRRRRPRRRGRRTGNTS		GGG	GVRRG	R	ETFVFS	K	41
NY-RPV	1	VVVVASN	GPARRGRRRRPVGPRRGR		T	PRSGGSRG		ETFVFS	K	42
BWYV	1	VVVVQTS	RATQRRPRRRRR	GNNRTGR	TVPTR	GAGS	S	ETFVFS	K	43
PLRV	1	VVMVTAS	GQPRRRRRRR	GGNRRSRR	TGVPR	GRGS	S	ETFVFT	K	42
A-PAV	1	VVVVQPN	RA GPRRRNGRR	KG RGGAN	FVFRPTGG		T	EVFVFS	V	41

MT-RMV	41	DSI	A	GSAPE	S	SPSGRLFLSAPA	FSG	GILKAYHEYKIT	KII	80
NY-RMV	42	DSL	T	GNAPG	S	SPSGRLYQSAH	FSS	GILKAYHEYKIS	KVT	80
NY-RPV	43	DSL	A	GNSSG	S	ITFGPSLSEYPA	FQN	GVLKAYHEYKIT	NCV	82
BWYV	44	DNL	A	GSSSG	A	ITFGPSLSDCPA	FSN	GMLKAYHEYKIS	MVI	83
PLRV	43	DNL	M	GNSQG	S	FTFGPSLSDCPA	FKD	GILKAYHEYKIT	SIL	82
A-PAV	42	DNL	K	ANSSG	A	IKFGPSLSQCPA	LSD	GILKSÝHRYKIT	SIR	81

MT-RMV	81	LEFISEASS	TQ	SGSIAYELDPHNK	LST	LASTINQISIVK	GGKGSISSKQ			129
NY-RMV	81	LEFISEASS	QS	EGSIAYELDPHNK	LSA	LSSTINEFSIVK	GGKKTLLSNQ			129
NY-RPV	83	LQFVSEASS	TA	AGSISYELDPHCK	ASS	LASTINKFTITK	TGARSFPAKM			131
BWYV	84	LEFVSEASS	QN	SGSIAYELDPHCK	LNS	LSSTINKFGITK	PGKRAFTASY			132
PLRV	83	LQFVSEASS	TS	SGSIAYELDPHCK	VSS	LQSYVNKFQITK	GGAKTYQARM			131
A-PAV	82	VEFKSHASA	NT	AGAIFIELDTACK	QSA	LGSYINSFTISK	TASKTFRSEA			130

MT-RMV	130	IGGGV	WHDSSSEDQFAI	LYKGGG	NSS	V	AGSFRIT	M	VHTQ	NPK	170
NY-RMV	130	IGGGV	WRDSTEDQFAI	LYKGGG	KSS	I	AGSFRVT	MDVLTQ		NPK	171
NY-RPV	132	INGLE	WHPSDEDQFRI	LYKGGG	ASS	V	AGSFKIT	LRVQLQ		NPK	173
BWYV	133	INGTE	WHDSSSEDQFRI	LYKGGG	SSS	I	AGSFRIT	ILCQFH		NPK	174
PLRV	132	INGVE	WHDSSSEDQCRI	LWKGGG	KSSDT		AGSFRVT	IRVALQ		NPK	174
A-PAV	131	INGKE	FQESTIDQFWM	LYKANG	TTTDT		AGQFIIT	MSVSIM		TAK	173

Fig. 23. Comparison of the deduced amino acid sequences of the MT-RMV Lu1-Lu4 genome fragment with the coat protein region of NY-RMV, NY-RPV, BWYV-FL1, PLRV-1 and Aus-PAV.

MT-RMV	1	FDWSVADWMLHDD	R?	VRNRLT	RNKNTATPSA	SCWLK CISNSVLCLS	46
NY-RPV	1	FDWSVSDWMLADD	ME	VRNRLT	IDCNELTRHLR	AVWLQGISNSVLCLS	47
BWYV	1	FDWSVADWMLHDD	MI	VRNRLT	IDLNPATERLR	SCWLRCISNSVLCLS	47
PLRV	1	FDWSVAYWMLEDD	ME	VRNRLT	FNNTQLTERLR	AAWLK CIGNSVLCLS	47
MT-RMV	48	DGTLA	?AH?RN	QKSGSYNTSSSNSRIRVM??	YHC	GATWAMAMGDDALES	97
NY-RPV	48	DGTMLA	QRVPGV	QKSGSYNTSSTNSRVRVMAA	YHC	GASWAIAMGDDALEA	97
BWYV	48	DGTLA	QIHPGV	QKSGSYNTSSSNSRIRVMAA	FHT	GAIWAMAMGDDALES	97
PLRV	48	DGRLA	QTVPGV	QKSGSYNTSSSNSRIRVMAA	YHC	GADWAMAMGDDALEA	97
MT-RMV	98	SRHGLDV	YKILGFKVEVSKQLEFCS	122			
NY-RPV	98	PDTDLSK	YKDLGFKVEVSGELEFCS	122			
BWYV	98	NPADLAA	YKKGFKVEVSGQLEFCS	122			
PLRV	98	PNSDLEE	YKTLGFKVEVGRELEFCS	122			

Fig. 24. Comparison of the deduced amino acid sequences of the MT-RMV Pol1-Pol2 genome fragment with the RNA dependent RNA polymerase region of NY-RPV, BWYV-FL1 and PLRV-1.

Discussion

PCR techniques have enabled us to easily distinguish between two serologically indistinct BYDV isolates without performing time and labor consuming aphid transmission tests. The use of PCR and selected restriction enzymes, for digest analysis, as diagnostic tools produced unambiguous results. The restriction enzyme mapping of the MT-RMV-V Lu1-Lu4 PCR fragment revealed the presence of band artifacts in the polyacrylamide gels. These artifacts may be the result of restriction enzyme digestion of extraneous PCR products. They may be extraneous PCR products which could not be cut by a particular restriction enzyme but which were still detectable. For instance, there was no restriction site in the MT-RMV-V RNA sequence that was recognized by the Hind III enzyme; yet, two bands were visible in the polyacrylamide gel lane containing this sample. A small band, found in both the Alu I and Eco RI digest sample lanes, appears to be an artifact. With Alu I digestion, a restriction fragment was too small to visualize in a gel. The restriction mapping for the NY-RMV Lu1-Lu4 piece correlated well with the observed fragments produced by restriction enzyme digest analysis. However, some bands were too small to visualize or represented two similarly sized fragments. By comparing known restriction fragment lengths determined from sequence data with observed bands

from actual enzyme digests, a better evaluation of a restriction enzyme for diagnostic purposes can be made. Sau 3A1 would be the best choice as a diagnostic restriction enzyme for distinguishing between the NY-RMV and MT-RMV-V isolates. There was a definite difference in the number and size of the bands produced for each Sau 3A1 digested sample. Hinf I would also be a good choice, because of the different sized fragments produced for each sample even though the same number of fragments were produced. Hind III would be a poor choice since no site is recognized by this enzyme. Alu I, Eco RI and Hae III individually produce fragments which are of similar size for both the NY and MT Lu1-Lu4 PCR products, and thus they would not be satisfactory for distinguishing between these two isolates.

The results of the Northern and Southern blots show that the use of radioactive probes, derived from labelling PCR fragments, can detect virus from MT-RMV-V and NY-RMV infected leaf material. The radioactively labelled MT-RMV-V Lu1-Lu4 PCR fragment was highly specific in detecting MT-RMV-V RNA. Its detection of NY-RMV RNA was slight. In Southern blot experiments, the radioactively labelled MT-RMV-V Lu1-Lu4 PCR product was less specific. Although it did hybridize to its homologous PCR products derived from MT-RMV and NY-RMV isolates and not to those derived from the other BYDV isolates, additional non-target bands were observed in the autoradiographic films. This may, however,

be the result of not purifying the PCR band prior to labelling. Radioactive probes may be necessary for more accurately determining the viral nucleic acid size in denaturing gels. Viral RNA from the MT-RMV-V isolate was difficult to size under denaturing conditions with ethidium bromide staining.

PCR methodology has allowed us to partially sequence the MT-RMV-V and the NY-RMV isolates in order to further characterize, and to a certain extent, delineate the two isolates. Although sequencing was only done for a small portion of the genome of the two isolates, these preliminary sequences apparently indicate that the MT-RMV-V and the NY-RMV isolates are more similar, at the molecular level, to BWYV, PLRV, and NY-RPV than to the Group I BYDV members. Vincent et al. (142) have reported nucleotide sequence identities for the NY-RPV isolate with BWYV, PLRV and the Australian PAV isolate. The NY-RPV isolate shows 65.4%, 69.4% and 55.2% nucleotide sequence homology with BWYV-FL1, PLRV-1 and Aus-PAV, respectively. The MT and NY RMV isolates show slightly higher sequence identities with BWYV-FL1 and Aus-PAV as compared to those shown by NY-RPV. The RMV isolates show slightly lower sequence identities with PLRV-1 than is shown by NY-RPV. The two RMV isolates still show more sequence identity between them (79.8%) than to the NY-RPV (57.1%). However, it would be premature to align the MT-RMV-V and NY-RMV isolates with BWYV, PLRV or

NY-RPV without the complete sequences being known. J. Vincent (Purdue University, Lafayette, IN, personal communication) has speculated that based on the sequence data for both the coat protein region and the RNA dependent RNA polymerase region, that the MT and NY RMV isolates would have a genomic organization similar to that of BWYV, PLRV and NY-RPV. Sequencing the rest of the genomes and a few of the other BYDV isolates should yield information regarding taxonomic relationships in the Luteovirus group. It would be of interest to sequence the NY-SGV isolate to compare with the MT-RMV-V sequence based on the greenbug aphid vector's ability to transmit both. Possibly a taxonomic realignment of all BYDV isolates will be needed.

CHAPTER 4

SUMMARY

The need for investigation of Montana barley yellow dwarf virus isolates arose with the intriguing problem associated with the 1978-1981 collection of apparent RMV-like isolates. They did not react with W.F. Rochow's bank of BYDV antisera. These initial Montana RMV-like isolates stimulated us to investigate this unusual situation and subsequently led to isolate collections in 1985 and 1986. Early experiments with the second collection were centered on aphid transmission efficiencies of the various isolates (15). It was found that these new isolates could now be detected serologically by Rochow's NY-RMV polyclonal antisera. It is uncertain whether the original isolates collected from 1978-1981 were truly serologically distinct or whether the antisera of the time was just not as efficient as currently produced antisera. However, a new finding was made. The Montana isolates could be vectored by both corn leaf aphids and greenbugs. NY-RMV is rarely transmitted by greenbugs. This finding enabled us to continue our investigation of the Montana isolates.

In this study, we were able to purify and partially characterize one particular MT-RMV isolate. Modification of

the buffer system used and the centrifugation steps of existing purification procedures allowed us to consistently produce purified virus preparations. Purification of RMV isolates in the past has usually been avoided by BYDV workers because of the notion that these isolates were difficult to purify. The procedure greatly enhances the virion yield when compared to previous methods (24,146). Our purification procedure enabled us to study the MT-RMV-V isolate. The virion diameters of the MT isolate were similar to those reported for the other BYDV isolates. Although estimations of the nucleic acid size and the coat protein size appear to be smaller than those values reported for other isolates, we believe they are comparable. It will only be after a complete sequencing of the MT-RMV-V genome that an accurate value for nucleic acid size and coat protein size can be determined. Vincent et al. (142) noted that actual coat protein sizes of three BYDV isolates, based on sequence data, were slightly smaller than those estimated by SDS-PAGE analysis.

Our investigation into the cytological alterations induced by MT-RMV-V infection in a plant revealed some interesting anomalies. In some respects, the induced changes were very similar to those changes induced by a Manitoba RMV isolate (45). These changes include the presence of massive clumps of virions in the cytoplasm, extensive callose cell wall deposits, the lack of densely

staining material filling the plasmodesmata and the presence of intact and normally shaped mitochondria in the phloem cells. A major alteration induced by the Manitoba RMV isolate that was not often seen in MT-RMV-V infected cells was the increased numbers of highly proliferated membranous tubules and spherical particles with radial arms. These changes were not commonly seen in RPV infected cells (47) either. In fact, the RPV and the MT-RMV-V isolate induced the production of rod-like, branched tubules that were similar for both isolates. These branched tubules were not found in the Manitoba RMV infected material (45). The variety of changes induced by the MT-RMV-V isolate makes sub-group classification based on cytological alterations more challenging.

Our work with polyclonal and monoclonal antibodies was not successful in regards to distinguishing between the serologically indistinct NY-RMV and MT-RMV-V isolates. But, we were successful in producing potentially useful diagnostic antibodies for RMV isolates in general.

Using PCR and restriction enzyme methodologies, we were the first investigators to unambiguously distinguish between two serologically indistinct isolates. The banding patterns of digested PCR genomic fragments were unique between these two isolates when certain restriction enzymes were used. The possibility of producing fragment artifacts with some restriction enzymes is high. An investigation of recognized

restriction sites of the known RNA sequence may be necessary before the choice of a diagnostic restriction enzyme should be made.

The PCR technique enabled us to characterize some of the molecular differences between the MT and NY RMV isolates. The partial nucleic acid sequences that we obtained give support to earlier hypotheses that the BYDV sub-group II members (RPV and RMV) do not belong in the BYDV group. Sequence homology and genomic organization of the sub-group II members appear to be more similar to those of BWYV and PLRV than to the BYDV sub-group I members (PAV and MAV). But, as stated earlier, the differences in aphid vectors and host range may preclude aligning the sub-group II members with either BWYV or PLRV (141). Sub-group II members induce symptoms in monocotyledonous plants which have always been attributed to the barley yellow dwarf virus. They do not infect dicotyledonous plants like BWYV and PLRV. Although the sequence data is still incomplete for the RMV isolates, our conclusion is that sub-group II should not be aligned with BYDV, but should be regarded as a distinct and separate virus with RPV and RMV as isolates of this virus.

Any future work on the MT-RMV-V isolate should center on the completion of nucleic acid sequencing and the comparison of that sequence to other RMV isolates. This may elucidate possible relationships among geographically

diverse RMV isolates and may give clues as to their success in those regions. Because barley yellow dwarf virus is a global problem, additional information on how these isolates can survive in and exploit the plants in a given region will be very important.

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