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Polyclonal antisera were raised against isolates of bean common mosaic virus (BCMV) and bean common mosaic necrosis virus (BCMNV) using conventional serological methods. Infected tissues containing, respectively, 22 recognized BCMV and BCMNV isolates were tested against the two antisera by antigen-coated plate (ACP) ELISA and double antibody sandwich (DAS) ELISA. Results indicated that each immunoglobulin was virus-specific by DAS-ELISA, providing clear distinction between BCMV and BCMNV.

A reverse transcription, polymerase chain reaction (RT-PCR)-based assay in combination with restriction endonuclease analyses, was developed for molecular detection of BCMV, BCMNV and their pathogroups. Specific detection of the two viruses was accomplished by constructing two virus-specific primer pairs that amplified a PCR product specific for each virus. Distinction of two BCMNV pathogroups (PG-III and PG-VI) was achieved by restriction enzyme *XbaI* digestion of BCMNV PCR

products. However, none of the tested restriction enzymes clearly differentiated the five recognized BCMV pathogroups. A primer pair Dts/Uny15 specific for BCMV pathogroup V was also developed. By its RT-PCR application, four BCMV-PG-V isolates were differentiated from the other known variants of BCMV pathogroup I, II, IV and VII. Thus, by a combination of RT-PCR and restriction enzyme analyses, it was possible to differentiate both viruses, and two pathogroups of BCMNV, and one pathogroup of BCMV.

Serological and Molecular Approaches for Distinguishing Bean Common Mosaic and Bean Common Mosaic Necrosis Potyviruses and their Respective Pathogroups

by

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Ling Xu, Author

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1. INTRODUCTION

Common bean (Phaseolus vulgaris) is grown worldwide in a broad range of cropping systems and under diverse environmental conditions. According to FAO reports (1975), the total world planting of bean crops in 1974 was more than 23 million hectares which exceeded potatoes in the same year, indicating the great importance of the crop.

Bean common mosaic virus (BCMV) was first described in United States in 1917 (Stewart and Reddick). The virus can cause severe damage to bean plants by inducing stunting, leaf mosaic and distortion, as well as lethal necrosis (See especially BCMNV, pp.2,3). BCMV is also distributed worldwide and has long been a major concern in producing both green vegetable beans and bean seed crops (Zaumeyer and Thomas, 1957).

At the outset of this research, nucleotide sequences had been completed in only the capsid protein coding region of three isolates of BCMV (Khan et al., 1993). Sequencing of the entire BCMV genome (for any isolate) would wait for two years, until work was completed in the laboratories of Drs. S. Wyatt and P.H. Berger, Washington State University and University of Idaho, Moscow, respectively. With only minimal BCMV sequence information available, I began exploring available molecular

methods that could differentiate pathogroups (groups of isolates representing the same pathotype) of BCMV. this same time, international efforts had begun to partition and re-name two seemingly different virus species formerly included under the term, BCMV. This endeavor came to completion at the very close of this thesis research. A paper by Mink et al (1994) named the new virus bean common mosaic necrosis virus (BCMNV), with the approval of the International Committee on the Taxonomy of Viruses.

Since the early 1960's, when Hubbeling noted severe BCMV-like symptoms, the virus now recognized as BCMNV was considered a severe variant of BCMV. This assumption is understandable, because both viruses comprised numerous pathogenic variants (i.e., variants determined by specific Phaseolus genes = pathotypes) characterized by overlapping abilities to attack bean genotypes. Other marked similarities between BCMV and BCMNV are identical transmission modes and virion morphology, a shared range of symptom types, and similar serological properties. A significant biological distinction between the two viruses is that BCMNV attacks bean cultivars of the I,I genotype (i.e., possessing the "dominant I" gene) and, therein, causes lethal temperature-independent systemic necrosis, sometimes referred to as black root (Zaumeyer and Thomas, 1948).

From the definitive pathology/genetic work of E. Drijfhout (1978), BCMV strain differentiation became well understood for the first time. This understanding was soon

applied to international breeding efforts for developing superior, BCMV-resistant bean cultivars.

Because two viruses were unknowingly encompassed in these genetic/breeding efforts, the same set of bean cultivars representing 11 virus-resistance groups were used to differentiate pathogroups of both BCMV and BCMNV. Thus, the pathogroups referred to by Drijfhout (1978) as BCMV pathogroup III and VI are now known as BCMNV pathogroups. All of the remaining pathogroups of Drijfhout are still retained as BCMV pathogroups. The pathogroup classification system has proven very successful, and remains in use, for monitoring and identifying "new" viral variants as well as for BCMV- and BCMNV- resistance breeding.

Several diagnostic and analytical approaches have been applied to BCMV and BCMNV detection and differentiation, including polyclonal antisera to viruses (Lana et al., 1988), and to N-terminal amino acids of viral capsid protein (Khan et al., 1990), monoclonal antibodies (Wang et al, 1984; Mink et al, 1992) and HPLC of partially digested capsid protein (McKern et al.,1992). The two viruses were clearly distinguished by most of these approaches except that polyclonal antisera had not succeeded in this purpose prior to the present study. However, identification of pathogroups was not resolved by such approaches and remains dependent on symptomatic reactions of defined Phaseolus genotypes (Drijfhout, 1978), use of which involves time/space-consuming, laborious effort and sometimes equivocal results.

Applications of current molecular approaches were viewed as an attractive alternative to the need for bean-cultivar differentials. For instance, the recent amplification of virus-specific nucleotide sequences by reverse transcription and polymerase chain reaction (RT-PCR) was used for specific detection and differentiation of pea seedborne mosaic potyvirus pathotypes (Kohnen et al 1992, 1995). Likewise, three tobamovirus pathotypes infecting L-resistant pepper genotypes were differentiated, using RT-PCR and restriction-enzyme analysis (Tenllado et al, 1994). Such approaches were not yet reported for the detection of BCMV and BCMNV.

The following, accordingly, were the objectives of this research. First, I proposed to reassess the ability of polyclonal antisera to detect and differentiate BCMV and BCMNV, using a new alternative adjuvant for antiserum production. I subsequently proposed to evaluate RT-PCR and restriction-enzyme analyses for possible differentiation of BCMV, BCMNV and their pathogroups.

2.LITERATURE REVIEW

2.1 Discovery, economic importance and nomenclature of BCMV

Bean Common Mosaic Virus has long been considered the most important seed-borne virus of common bean (Phaseolus vulgaris) (Zaumeyer and Thomas, 1957). The virus was first reported under the name bean mosaic virus by Stewart & Reddick in 1917. Early workers also used such terms as bean virus 1, phaseolus virus 1 and common bean mosaic virus when referring to this virus (Pierce, 1934; Martyn, 1968). The term bean common mosaic virus has become officially adopted since the 1970's (Bos, 1971). The virus is now known to occur worldwide, attributable primarily to inadvertent international trade or exchanges of BCMV-infected bean seed.

In the early 1900's, the bean common mosaic (BCM) disease was endemic wherever beans were grown because BCMV-free seedlots were unknown and seed-borne inoculum was field-spread by aphid vectors (Zaumeyer and Thomas, 1957). In instances of server BCM epidemics, the incidence of infected plants sometimes reached 100% by mid-bloom (Stewart, 1917). Hampton (1975) reported that moderate and severe BCM could cause, respectively, 50% and 64% reduction in the number of pods per plant, which in turn resulted in 53% and 68% reductions in seed yield. Moreover, seed harvested from BCMV infected plants may be disqualified by quarantines and rendered unmarketable (Drijfhout, 1978; Forst et al., 1991).

High incidences of BCMV were also reported in various P. vulgaris germplasm collections. Klein et al (1988) reported that 60% of the 13,000 accessions of USDA P. vulgaris germ plasm collection was contaminated with BCMV. A similar level of BCMV infection was also found in the world P. vulgaris collection center at the Centro Interncional de Agricultura Tropical (CIAT) in Cali, Colombia (Klein et al, 1990). In response to this serious problem, BCMV elimination programs were undertaken at the Phaseolus germ plasm repositories in both USDA (Klein et al, 1986) and CIAT (Klein et al., 1990).

In the United States, use of certified seed and resistance breeding efforts have helped to minimize epidemics of BCM from 1930 to the middle of 1970's (Provvidenti et al., 1983). However, in the late of 1970's and 1980's, outbreaks of multiple strains (Hampton et al., 1983) and of the African necrotic strain of BCMV (now designated BCMNV) in Idaho (Myers et al., 1989), Michigan (Silbernagel et al., 1983; Kelly et al., 1983) and New York (Provvidenti et al, 1983) caused the re-emergence of BCMV as a major concern. The African necrotic strain (or BCMNV) was reported to attack many commercial cultivars considered to be resistant to BCMV commercial cultivars such as Pinto UI 114, UI 126, NW410 and Great Northern UI 60. The Idaho Crop Improvement Association rejected about 650 acres of bean seed crops in 1989 because of infection by this strain (Forster et al., 1991).

2.2 Host range, transmission and taxonomic position of BCMV

The host range of BCMV includes some 100 species of 44 genera of nine families (McKern et al., 1992). In most cases, however, P. vulgaris cultivars are the only economic hosts of BCMV (Drijfhout, 1978). Natural infection of Yellow lupin by BCMV was reported in Poland (Frencel and Pospieszny, 1979), of Rhynchosia minima in Colombia (Meiners et al., 1978) and of an unidentified legume weed in Africa (Spence and Walkey, 1991).

BCMV is transmissible in nature through seed, by pollen, and by several aphid species such as the pea aphid (Acyrthosiphon pisum), bean aphid (Aphis fabae) and green peach aphid (Myzus persicae) (Bos, 1971). Seed transmission rates were reported to range from 0 to 83% depending on cultivars, virus strains and stage of growth at the time of infection (Bos, 1971). Although BCMV was detectable in the embryo and cotyledon, little or no BCMV seed transmission occurred when mother plants became infected after flowering (Bos, 1971). BCMV can survive in the seed for at least 30 years (Pierce and Hungerford, 1929)

BCMV particles are flexuous filaments about 750 nm long and 15 nm wide (Bos, 1971). Based on particle morphology, mode of transmission and biological properties, Bos (1971) characterized BCMV as a member of potyvirus. The genome of potyviruses is known to comprise a single positive-sense stranded RNA, approximately 10 kb in length, containing one

open reading frame encoding a large polyprotein that is post transcriptionally processed (Dougherty and Carrington, 1988).

2.3 Strains and pathogroups of BCMV

From the early 1940's to late 1970's, more than 20 BCMV strains were reported, particularly in connection with P. vulgaris BCMV-resistance breeding. In 1978, through detailed studies of the genetic interactions between P. vulgaris and BCMV, E. Drijfhout established a standardized international set of BCMV bean differentials. By this work, he classified 22 previously described BCMV strains into seven pathogroups designated PG-I through PG-VII. Using these BCMV differentials, Schmidt et al (1987) identified all seven pathogroups from isolates collected from the German Democratic Republic and USSR and four pathogroups (PG-III, IV, VI and VII) from Hungary. All BCMV pathogroups except PG-III were also detected in the USDA Phaseolus germ plasm collection (Klein et al., 1992). Hampton (1983) determined that BCMV isolates associated with 1977 and 1981 BCM epidemics in the northwestern U.S.A. were members of PG-I, II, III, IV, V and VII, from which he concluded that some BCMV strains previously reported in Europe may have occurred for the first time in the United States. To date, it is generally accepted that Drijfhout's treatise has provided the most functional and accepted means for identifying BCMV pathotypes.

Based on BCMV-induced symptoms on certain host cultivars, Drijfhout (1978) also separated BCMV pathotypes or isolates

into three broad categories: mosaic-inducing strains, temperature-dependent necrosis-inducing strains and temperature-independent necrosis-inducing strains. The last two groups can both induce systemic necrosis symptoms (black root) on bean cultivars processing the dominant I gene (i.e. I,I genotype), but one group induces necrosis only at high temperature (30-35 C), whereas the other induces such symptoms irrespective of temperature. The systemic necrosis symptom consists of apical and stem necrosis, which may result in premature death of plants. The mosaic-inducing strains cause typical common mosaic symptoms, sometime associated with leaf malformation, but without inducing necrotic symptom on any cultivars. Drijfhout (1978) placed the temperature independent necrotic strains in PG-III and PG-VI. Whereas, the mosaic inducing strains together with temperature sensitive necrotic inducing strains were placed in the remaining pathogroups (PG-I, II, IV, V and VII).

2.4 Serology, molecular biology and relationship to other potyviruses

Although BCMV comprised a large number of strains with complex relationships, it was also found either serologically or biologically related to a number of other potyviruses. Recent serological and molecular biological studies (see below) have focused on clarifying relationships among BCMV strains and with other potyviruses.

Using antisera to three BCMV isolates (BCMV-NL1, -NL3 and

NY15) and a blackeye cowpea mosaic virus (BlCMV) strain W, Lana et al (1988) concluded that the BCMV mosaic-inducing strains NL1, NY15 and BlCMV strain W were closely related serologically. Those strains were distinguishable, however, from temperature independent necrosis-inducing strain NL3. By producing antibodies to N-terminal peptide of coat proteins of the above four strains, Kahn et al (1990) were able to more clearly distinguish strain NL3 from NL1 or NY15.

Vetten et al (1992) extended the serological study to 13 BCMV isolates spanning seven BCMV pathogroups. Their results indicated that the BCMV isolates could be grouped into two serogroups, A and B. Serogroup A included isolates NL3, NL5 and NL8 which were all temperature-independent necrosis-inducing strains. Serogroup B contained isolates of NL1, NL2, NL4, NL6, US4, NL7, NY15 and Florida which were either mosaic or temperature-dependant necrosis-inducing strains.

Mink et al. (1992) tested 18 BCMV isolates and some isolates of BlCMV, cowpea aphid-borne mosaic virus (CABMV), azuki bean mosaic virus (AzMV) and peanut stripe virus (PStV) by indirect ELISA against a panel of 13 monoclonal antibodies (MAbs) raised against the above viruses. Three MAbs reacted only to Serogroup A isolates, and 4 MAbs reacted to all Serogroup B isolates as well as isolates of BlCMV, AzMV, and PStV.

McKern et al (1992) compared 22 BCMV isolates along with BlCMV and PStV by high-performance liquid chromatography (HPLC) of coat protein peptide and concluded that two distinct

groups of viruses existed among those isolates. Isolates NL3, NL5, NL8 and TN-1 which induced temperature-independent necrosis symptoms comprised one group and isolates inducing mosaic symptoms and temperature-dependent necrosis, together with BlCMV, AzMV and PStV, comprised another group. The two groups corresponded respectively with Serogroups A and B.

The search for sequence data to settle the taxonomic position of BCMV isolates was investigated by Khan et al in 1993. They determined that the coat proteins of BCMV isolates NL1 and NY15 and a BlCMV isolate W were identical in size (287 amino acids) and exhibited 84 to 98% sequence identity in the N-terminal region, whereas the coat protein of BCMV isolates NL3 had only 261 amino acids and shared only 46 to 61% homology with NL1, Ny15 and W in the N-terminal region.

2.5 Current taxonomic considerations for BCMV and BCMNV

Based on the above results, particularly following the results of McKern et al (1992) and of coat protein sequence information obtained by Khan (1993), it became generally recognized that the temperature independent necrotic strains of "BCMV" (i.e., NL3, NL5, NL8 and TN1) should be considered as a distinct potyvirus species. The names of bean necrosis virus, bean necrotic mosaic virus and bean black root virus were proposed by several authors (Mckern et al., 1992; Vetten et al., 1992; Khan et al., 1993). In 1993, at the workshop on Potyvirus Taxonomy, the term bean common mosaic necrosis virus was proposed for this group of isolates. There was also

general agreement that the term BCMV should be retained and should apply to BCMV Serogroup B isolates, and to AzMV, BlCMV and PStV as previously suggested by McKern et al (1992) and Khan et al (1993).

3. MATERIALS AND METHODS

3.1 Detection of BCMV and BCMNV by polyclonal antibody serology

3.1.1 Virus isolates, propagation and purification

Isolates of BCMV and BCMNV used, their identity and sources are shown in Table 1. The pathogroup identity of each isolate was confirmed in this study by differential host testing as described by Drijfhout (1978). During these conformations, isolates showing discrepancies relative to original pathotype descriptions were assigned with the correct identity based on my results.

All viral isolates were maintained and propagated in the BCMV-susceptible P. vulgaris cultivar Red Kideney under standardized green house conditions (i.e. 15-32 C. with supplemental sodium vapor or fluorescent lighting as required). Virus isolates were purified (as outlined in Appendix 1) from infected tissues two weeks after inoculation. Isolates purified for antiserum production, were subjected to two cycles of CsCl gradient centrifugation.

3.1.2 Preparation of antisera, immunoglobulin G (IgG) and IgG-alkaline phosphatase (IgG-AP) conjugates

BCMV isolate TS and BCMNV isolate NL3 were selected for antiserum production. Antiserum was prepared by immunizing 6-week-old female New Zealand white rabbits. Initially, 100 ug of purified virus in 0.5 ml PBS buffer (pH 7.5) was mixed in

Table 1. Viral isolates, their identities and sources

Virus[a]	Isolate	Patho-group[b]	Source[c]
BCMV	Type	I	MJS
	TS	I	ROH
	NL1	I	MJS
	Iran	I	MJS
	PV25	II	MJS
	NL7	II	MJS
	Florida	IV	MJS
	Western	IV	MJS
	NL6	IV	MJS
	ID123	IV	MJS
	NW63-108[d]	IV	MJS
	NY15	V	ROH
	NY15(Prov)	V	MJS
	NY15(Dean)	V	MJS
	NY15(Zau)	V	MJS
	Mex	VII	MJS
	NL4	VII	MJS
BCMNV	NL8(Drij)	III	MJS
	NL8(Bash)	III	ROH
	TN1	VI	MJS
	NL3	VI	MJS
	NL5	VI	MJS

[a] BCMNV was formerly considered to be temperature independent necrosis inducing variant of BCMV

[b] See Drijfhout, 1978

[c] MJS = M.J. Silbernagel, USDA-ARS, Prosser, WA
ROH = R.O. Hampton, USDA-ARS, Corvallis, OR

[d] Original pathotype description of isolate NW63-108 was PG-VII.

a vial of TiterMax #R-1 adjuvant (CytRx Corporation, Norcross, CA.), finally comprising a water-in-oil emulsion. The emulsion was injected intradermally into 20 sites on both sides of the rabbit's back, after depilation. Two weeks later, a second intramuscular injection of 500 ug purified virus emulsified in incomplete Freund's adjuvant was administered. The rabbits were bled twice by cardiac puncture at 4 weeks and 6 weeks after the first injection. The blood samples were allowed to clot overnight at 4 C and the sera were harvested, aliquotted, and stored at -20 C.

Only antiserum from the first bleeding was used to prepare immunoglobulin G (IgG). Having been diluted 1:1 with double distilled water, the antiserum was combined with an equal volume of 36% sodium sulfate and the resulting protein precipitate was centrifuged at 12,000 g for 15 min. The pellet was resuspended in 18% sodium sulfate and again centrifuged. The final pellet was resuspended in 1 ml of phosphate buffered saline (PBS) buffer (pH 7.0) and dialyzed against three changes of 1 L PBS buffer. The concentration of IgG was estimated by absorbance at 280 nm (e.g. A_{280} 1.4 = 1 mg/ml). IgG-alkaline phosphatase (IgG-AP) conjugate was prepared by adding 1000 units of alkaline phosphatase to 1 ml IgG stock (i.e., 1 mg/ml). The mixture was dialyzed against 1 liter of PBS three times, and fresh glutaraldehyde was added to a concentration of 0.2%. This mixture was dialyzed in PBS buffer to remove unbound glutaraldehyde, producing the final preparation of IgG-AP.

3.1.3 ELISA standardization and specificity tests

The antigen-coated plate (ACP) enzyme-linked immunosorbent assay (ELISA) and double antibody sandwich (DAS) ELISA methodologies (see appendix 2) were used in these studies. A checkerboard titration test was initially carried out with homologous antigen to optimize dilutions of each reagent required for ACP- and DAS- ELISA formats. Using standardized ELISA, fresh or desiccated leaf tissues infected, respectively, with 22 viral isolates (Table 1) were tested against each antiserum or IgG in each ELISA format. The resulting ELISA absorbance values (A_{405}) provided the basis for determining serological distinctions among isolates.

In tests, each ELISA microtiter plate contained test samples, plant tissues containing homologous antigen, plant tissues that were free of virus, and extraction buffer. ELISA data comprised absorbance (A_{405}) values reflecting the presence of homologous virus present in test samples and each of the three test controls. Absorbance values were considered as either positive or negative reactions on a non-statistical comparison of infected plant and healthy-plant controls. Typically, any absorbance values of 0.1 or greater were considered as indications that homologous virus was present in the test samples. Tissue extracts of infected plant with well developed symptom typically produce absorbance values in excess of 2.0 after a 4-hour incubation period at room temperature. In that same time period , healthy-plant samples

usually produced absorbance values of less than 0.05, in comparison to 0.00 values for the buffer control.

3.2 Detection and differentiation of BCMV, BCMNV and their pathogroups by RT-PCR and restriction endonuclease analysis

3.2.1 RNA preparation

RNA was prepared from infected leaf tissues or purified virus essentially as described by Kohnen et al (1992). In brief, less than 0.1 g of infected tissue was macerated in liquid nitrogen with mortar and pestle. The resulting powder was immediately incubated for 45 min at 37 c in 1 ml extraction buffer (2.0 mM Tris-HCl (pH 8.2), 2.0 mM EDTA, 2% SDS and 0.1 mg proteinase K). The mixture was then extracted with an equal volume of Tris-equilibrated phenol. The nucleic acid was precipitated from the aqueous layer with 2.8 volume of ethanol and 0.4 volume of 5 M ammonium acetate at -70 C for at least 30 min. After being centrifuged at 10,000 g for 15 min and washed with 70% ethanol one or two times, the RNA pellet was dried under vacuum and resuspended in water. The RNA was stored at -20 C until used. Viral RNA, extracted from purified virus, was used as controls in RT-PCR assays of infected-tissue samples.

3.2.2 Primer design

Initially, a pair of non-specific primers Db/Udg (Table 2), aimed at amplifying an unknown sequence region from both

BCMV and BCMNV genomes was constructed according to conserved sequences in the polymerase coding region of several potyviruses (GenEMBL Database Accession Nos. x676673, M96425, M92280,D10930, D00441, S42280, M15239) and coat protein coding region of certain BCMV isolates (GenEMBL Database Accession Nos. L11890, L21767, L15332, L12740, L19539, Z17203). The region amplified by Db/Udg relative to potyviral genome and previously published BCMV sequence data is shown in Figure 1b. Two restriction sites, *XbaI* and *PstI*, were also incorporated into two primers in order to facilitate the subsequent cloning processes.

After partial sequences were determined for selected isolates of BCMV (TS, NY15, NL7, Florida and Mex) and BCMNV (NL3), two virus specific-primers (Dbcmv/Ubcmv, Dnl3/Unl3) and one pathogroup specific-primer set (Dts/Uny15) were designed from these sequences. The primer sequence, the expected PCR product size and positions respectively are shown in Table 2 and Figure 1c. Primer pair Dts/Uny15 was putatively specific for BCMV-PG-V. However only primer Uny15 was specific for PG-V; the sequence of Dts was found to be conserved in other isolates. The Uny15 sequence contained only a single nucleotide difference between NY15 and other isolates at its 3′ end.

3.2.3 Reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription was performed in a 20 ul reaction

Table 2. Primer descriptions

Primer[a]	Sequence[b]	Target	Size
Db	5′-ACTCGCCCTGCAGAGCATTGTACC-3′	BCMV+BCMNV	1,000
Udg	5′-GYGGNCARCCWTCTAGAGTKGTKGAYA AYACHYTOATGG-3′		
Dbcmv	5′-ACCACGCTGCAGCTAAAGAGAACA-3′	BCMV	1,450
Ubcmv	5′-AATCTAGATGATATCATACTCTCTA-3′		
Dnl3	5′-GAATTGAAAGCGTACTATCTAATACAG-3′	BCMNV	900
Unl3	5′-CAGCTTGAATTTGATTCTGATGATGAGGTG-3′		
Dts	5′-TGCAGTGTGCCTTTCAGTATTCTCGCT-3′	BCMV-PG-V	1,050
Uny15	5′-TATACAAGTGGACGGAG-3′		

[a] D = downstream primer; U = upstream primer

[b] Litter code for nucleotide at degenerate position: Y=C,T; R=A,G; W=A,T; K=G,T; H=A,C,T; O=G,C,T; N=A,C,G,T

Inserted restriction sites are underlined. *XbaI* site: TCTAGA; *PstI* site: CTGCAG.

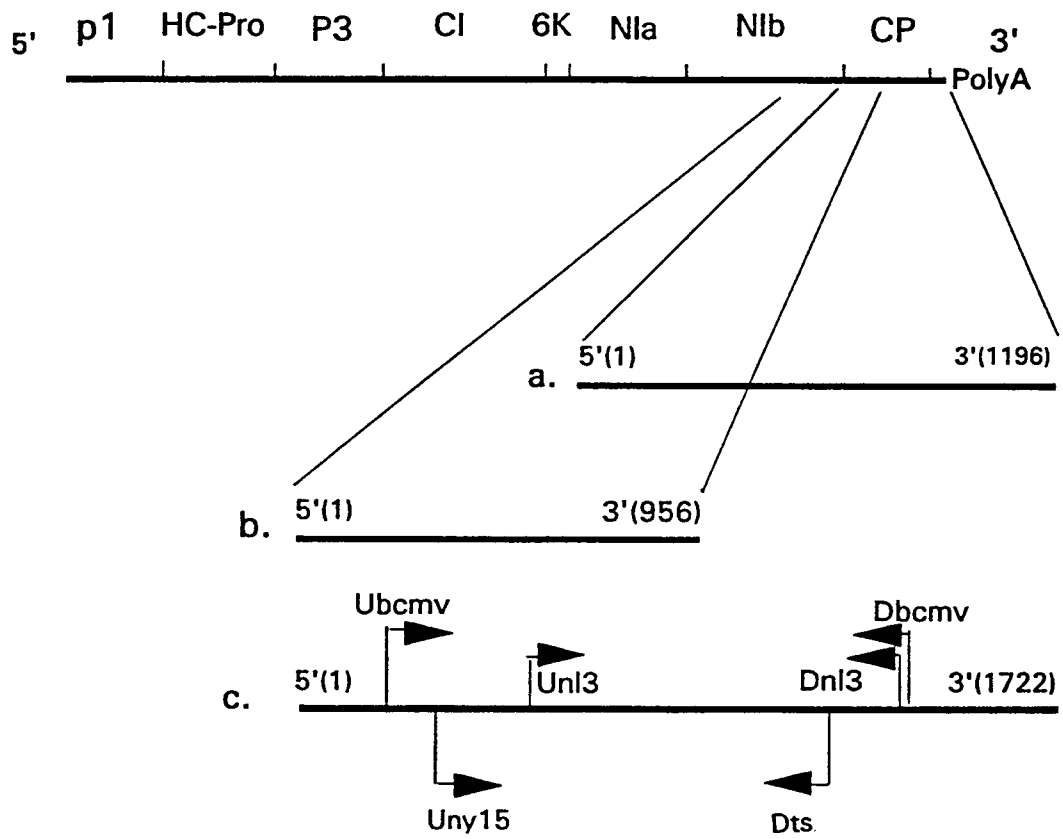


Figure 1. A consensus potyviral genome map. a. The region of BCMV genome in which sequence data was previously published; b. The region of BCMV genome in which sequence data was developed in these studies; c. Positions of constructed primer pairs.

mixture containing 50 mM Mg_2Cl, 10 mM Tris-HCl (pH 8.3), 1 mM each dNTP, 20 U RNase Inhibitor, 50 U M-MLV reverse transcriptase, 100 pM downstream primer and less than 1.0 ug RNA. The mixture was overlaid with 75 ul of mineral oil and incubated at 42 C for 1 hour, and then heating to 99 C for 5 min to inactive the reverse transcriptase.

PCR was performed in the same tube with 100 ul reaction mixture containing 50 mM KCl, 2 mM Mg_2Cl, 10 mM Tris-HCl (pH 8.3), 0.2 mM each dNTP, 100 pM of upstream primer and 2.5 U Taq DNA polymerase. Thirty-five reaction cycles were repeated, with 1 min period at 37 C for annealing, 1 min at 72 C for extension and 1 min at 94 C for melting.

Aliquots (8 ul) of PCR amplification product were analyzed by electrophoresis on 1.2% agarose gels stained with 0.5 ug/ml ethidium bromide.

3.2.4 Amplification, cloning and sequencing of RT-PCR products

Five BCMV isolates (TS, NY15, NL7, Florida and Mex) and one BCMNV isolate (NL3) representing 6 distinct pathotypes were selected for amplification with primer pair Db/Udg. After gel electrophoresis, bands representing predicted PCR products were isolated from the gel by the Prep-Gene kit (Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond, CA 94804), and subsequently cloned into the *XbaI-PstI* sites of plasmid pUC118 and pUC119 as described by Maniatis (1982). Clones shown to have the correct insert were sequenced with forward

pUC/M13 primer (5'tgtaaaacgacggccagt3') by the Service Laboratory of the Center for Gene Research, Oregon State University. Sequence assembly and alignment was accomplished using the GCG (Program Manual for the GCG Package, Version 7, April 1991, 575 Science Drive, Madison, Wisconsin, USA) and the IG (IntelliGenetics, Inc) software package.

3.2.5 Restriction endonuclease analysis

PCR products generated from the two virus-specific primer pairs were subjected to restriction endonuclease analysis. Four restriction enzymes *XbaI*, *EcoRI*, *BamHI* and *EcoRV* were used.

Restriction enzyme digestion was performed overnight at 37 C in 8 ul reaction mixture containing 5 ul PCR product solution, 0.8 ul 10x restriction enzyme buffer, 2.2 ul water and 2-5 units of restriction enzyme. The digestion products were resolved in 1.2% agarose gels by electrophoresis.

4. RESULTS

4.1 Detection of BCMV and BCMNV by polyclonal antibody serology

4.1.1 Yields of purified virus and immunoglobulin G (IgG)

An average yield of 10 mg purified virus was obtained from 100 g of infected Red Kidney plant tissue for both viruses. Dilutions of purified virus were monitored spectrophotometrically and exhibited 260 nm : 280 nm absorption ratios of 1.25. The average IgG yield from 1 ml of antiserum was 5.74 mg for BCMV and 3.92 mg for BCMNV.

4.1.2 ELISA Standardization

DAS-ELISA checkerboard titration indicated that optimal concentrations of reagents were 400 ng per ml of homologous IgG and an equivalent concentration of IgG-alkaline phosphatase conjugate (i.e., 2,500-fold dilution of IgG-AP conjugate), for detecting BCMV or BCMNV in plant tissue extracts with their homologous polyclonal antibodies. The IgG to BCMV and BCMNV did not react with healthy tissue extracts at those concentrations.

In ACP-ELISA, the optimal dilutions of antisera for detecting the homologous antigen in infected plant sap were 25,000x for BCMV and 5,000x for BCMNV. BCMV antiserum reacted weakly with extracts from healthy plant tissues in this ELISA format. This reaction was eliminated by cross absorption of

the antiserum with extracts of healthy plant tissue (1g/50 ml buffer) before use.

4.1.3 Serological specificity

Results from tests of two antibodies against 22 viral isolates in both ACP- and DAS-ELISA formats are presented in Table 3. In DAS-ELISA, anti-BCMV IgG reacted with all 17 BCMV isolates (A_{405}, 0.24-3.00), but not with any isolate of BCMNV (i.e., A_{405}, 0.00). BCMNV antibody also reacted only with BCMNV isolates (A405, 1.26- 3.00), and not with isolates of BCMV (i.e., A_{405}, 0.00-.01). The range in ELISA A_{405} values among isolates of each virus generated by both antibodies appeared not to be correlated with viral pathogroups.

In ACP-ELISA, the respective antibodies reacted with all isolates of the homologous virus, but also cross-reacted between viruses: anti-BCMV antiserum, A_{405}, 0.01-0.32; and anti-BCMNV antiserum, A_{405}, 0.02-0.80. Thus, serological specificity was obtained only with DAS-ELISA.

4.2 Detection and differentiation of BCMV, BCMNV and their pathogroups by RT-PCR and restriction endonuclease analysis

4.2.1 Sequence analysis of RT-PCR products

Predicted 1-kb PCR products were amplified from all six isolates (TS, NY15, NL7, Florida, Mex and NL3) with primer pair Db/Udg. Nucleotide sequences of the 1-kb products of six viral isolates are presented in Figure 2. Among the isolates,

Table 3. Detection of BCMV and BCMNV with their respective polyclonal antibodies in two ELISA formats

Virus	Pathogroup	Isolate	A_{405} value DAS-ELISA BCMV (Ab)[a]	DAS-ELISA BCMNV (Ab)	ACP-ELISA BCMV (As)	ACP-ELISA BCMNV (As)
BCMV	I	TS	3.00	.00[b]	3.00	.23
		NL1	.29	.00	.19	.02
		Iran	2.34	.00	1.48	.08
		Type	.82	.00	2.43	.10
	II	PV25	2.61	.01	3.00	.07
		NL7	1.15	.00	.38	.04
	IV	Western	1.29	.00	1.74	.38
		Florida	.69	.01	2.10	.48
		NL6	.31	.00	.39	.07
		ID123	3.00	.00	3.00	.41
		NW63-108	1.09	.00	.49	.05
	V	NY15	.49	.00	1.21	.07
		NY15(Prov)	.39	.00	.89	.18
		NY15(Dean)	.85	.00	2.51	.80
		NY15(Zau)	.31	.00	.24	.02
	VII	Mex	.24	.00	.95	.19
		NL4	.41	.00	.69	.06
BCMNV	III	NL8(Bash)	.00	3.00	.32	1.66
		NL8(Drij)	.00	3.00	.40	.54
	VI	NL3	.00	3.00	.01	3.00
		NL5	.00	3.00	.13	3.00
		TN1	.00	1.26	.17	3.00
		Healthy -plant	.00	.00	.00[c]	.00

[a] Ab = antibody. As = antiserum

[b] When A_{405} value was less or equal zero, the A_{405} value was scored as zero.

[c] Antiserum cross-absorbed with healthy-plant extract

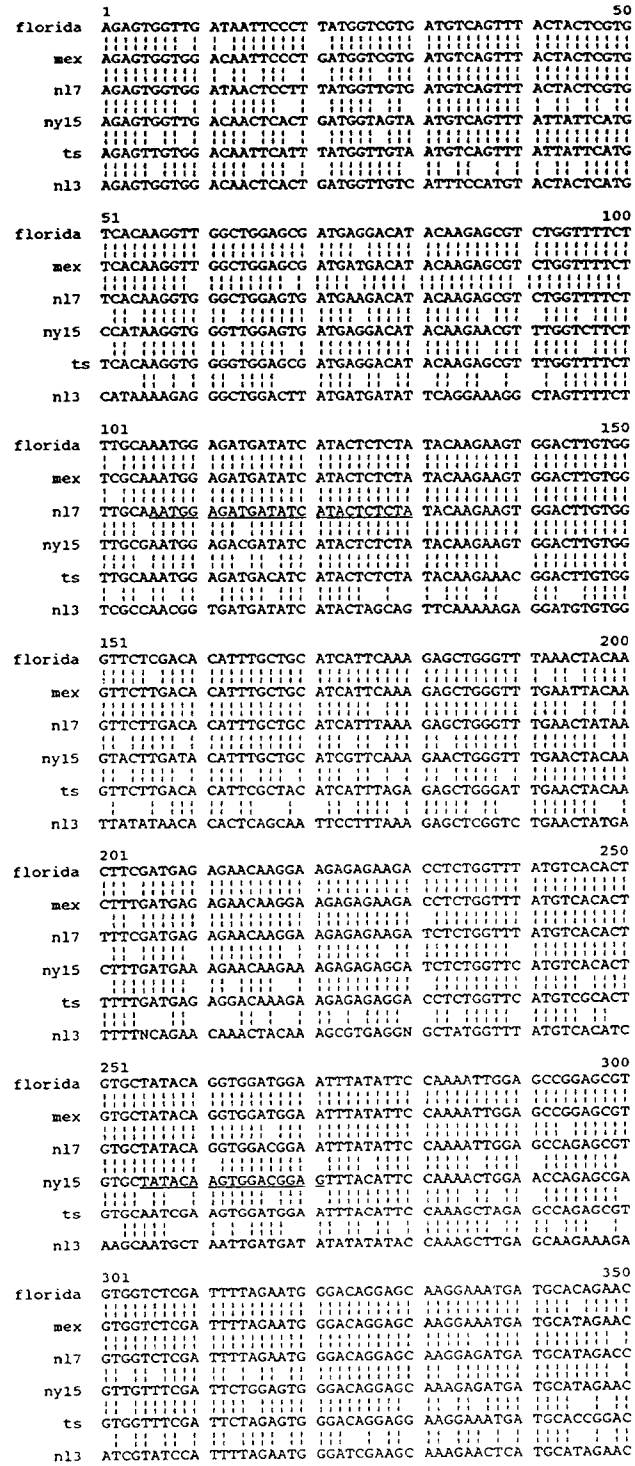


Figure 2. Alignment of nucleotide sequences of RT-PCR products generated from BCMV isolates TS, NY15, NL7, Florida and Mex and BCMNV isolate NL3. The position of upstream primer Ubcmv, Uny15 and Unl3 are underlined.

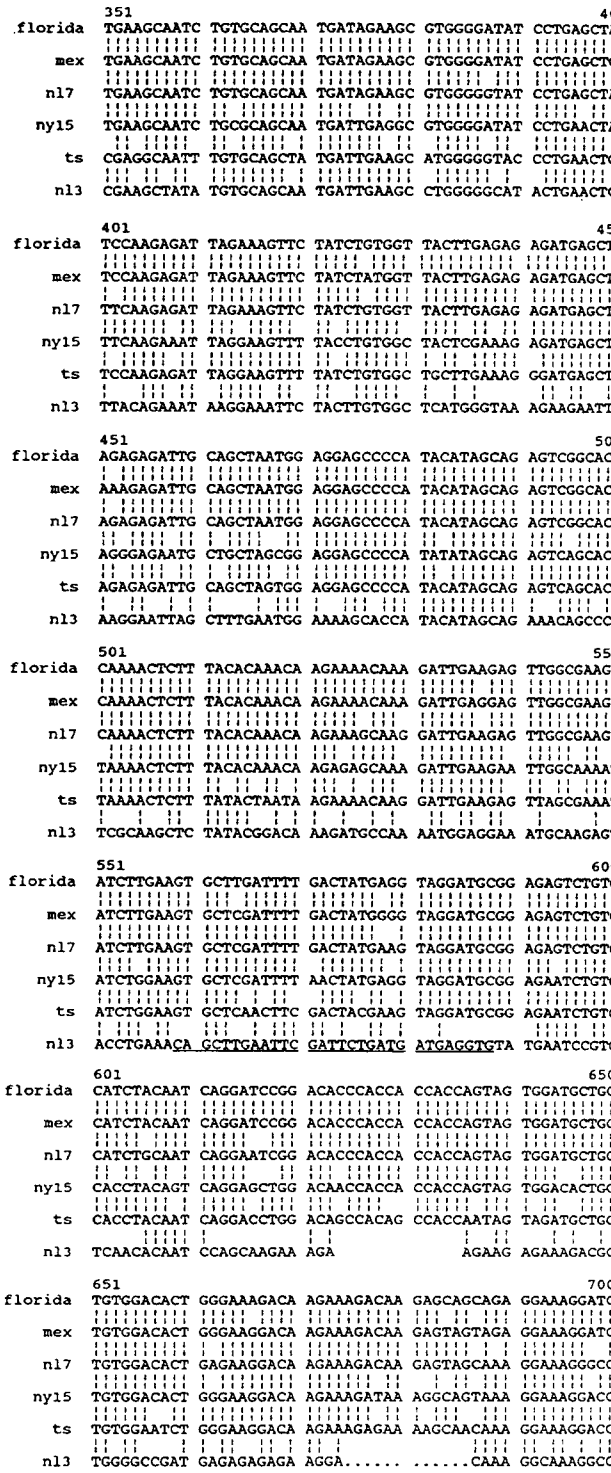


Figure 2. (continued)

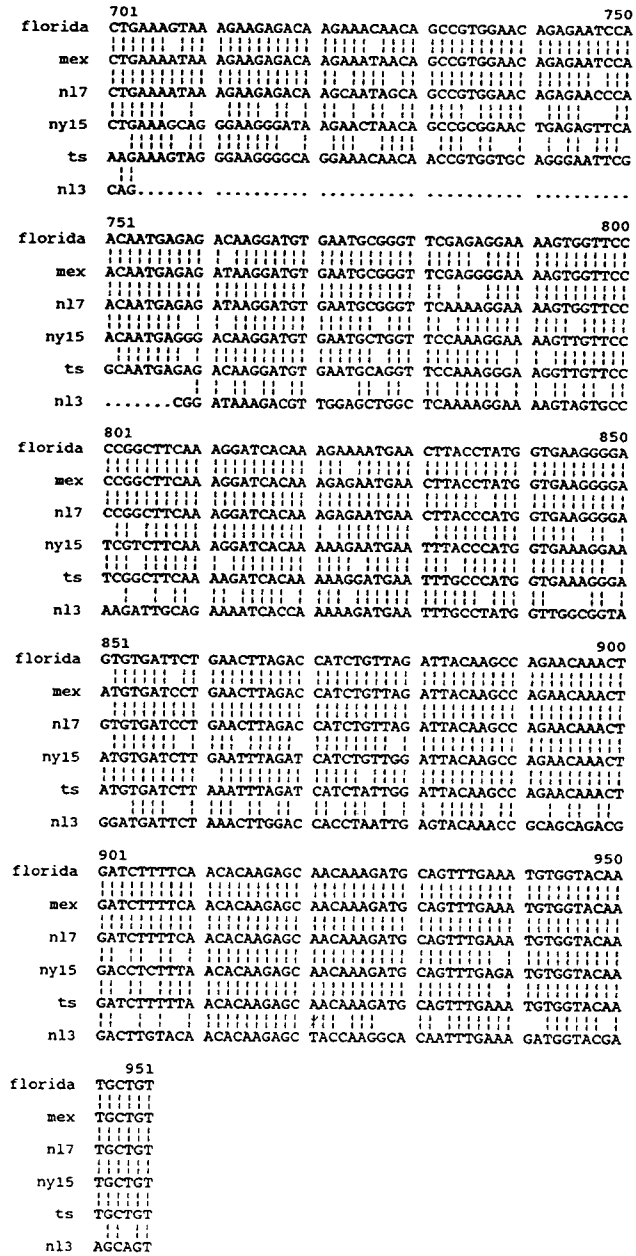


Figure 2. (continued)

sequences from 5 BCMV isolates were highly conserved both in length (956 bp) and in homology (86% to 97 %). BCMNV isolate NL3 was less conserved relative to BCMV, with 879 bp and 65% to 67% homology. Comparisons of the putative amino acid sequence (Figure 3) revealed that the most diverse region between BCMNV-NL3 and BCMV isolates(i.e., pathogroup representatives) was in the N-terminal regions of coat proteins (CP). This region of NL3 comprised 26 amino acids compared to 52 amino acids of BCMV isolates, with 46% to 58% homology with that of BCMV isolates (Table 3). Interestingly, the conserved motif DAG which was previously reported to be required for aphid transmission, occurred in the CP N-terminal of all isolates except BCMV-NY15.

4.2.2 RT-PCR amplification with specific primer pairs

Twenty-two isolates of BCMV and BCMNV (Table 1) were subjected to RT-PCR amplification with 3 specific primer pairs (Table 2) using nucleic acid extracts from infected plant tissue. Primer pair Dbcmv/Ubcmv generated a predicted 1450 bp product from all 17 BCMV isolates. No detectable PCR products were amplified from tissue infected with BCMNV isolates, in parallel tests (Figure 4A). Similarly, primer pair Dnl3/Unl3 amplified a predicated 900 bp product exclusively from all BCMNV isolates (Figure 4B). Primer pair Dts/Uny15 amplified a 1050 bp DNA product from all four isolates of BCMV-PG-V (NY15, NY15(prov), NY15(dean) and NY15(zau) but not from other BCMV pathogroups (Figure 4C). None of the primer pairs

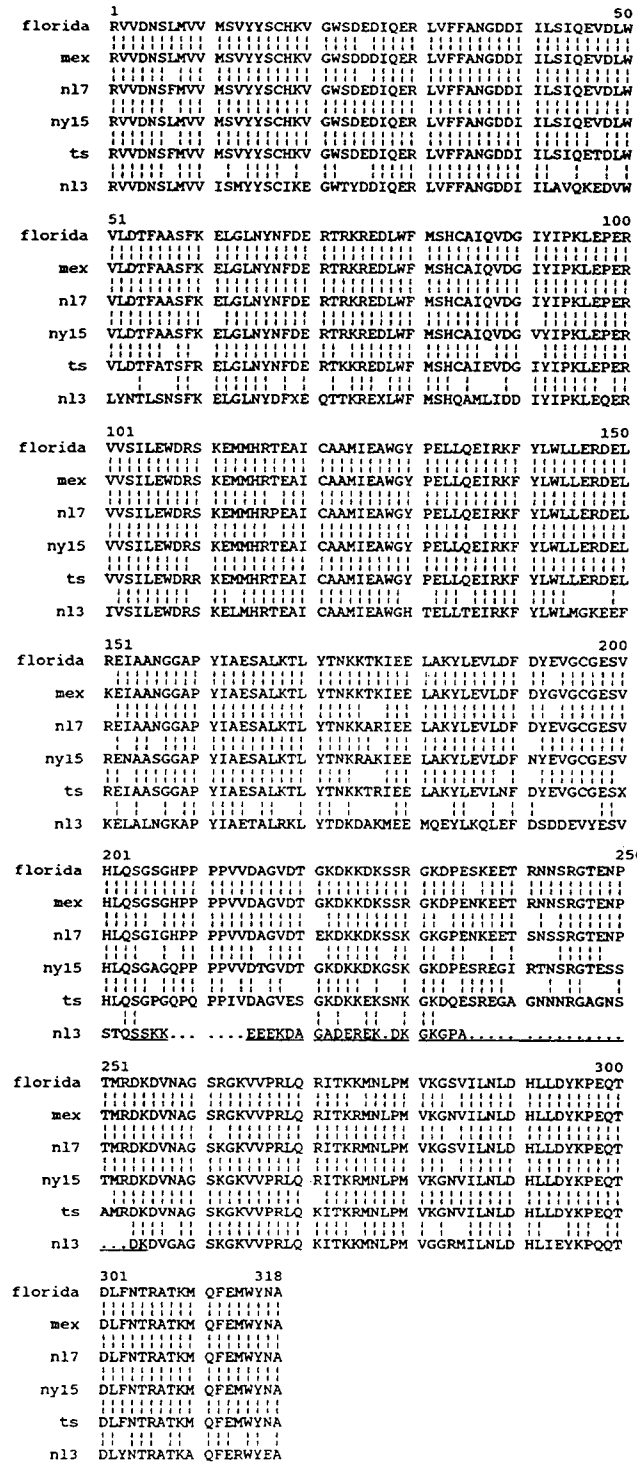


Figure 3. Alignment of putative amino acid sequences of RT-PCR products generated from BCMV isolates TS, NY15, NL7, Florida and Mex and BCMNV isolate NL3. The N-terminal regions of the coat protein are underlined (Shukla et al, 1988).

Table 4. Homologies (%) of putative amino acid sequences of RT-PCR products generated from BCMV isolates TS, NL7, NY15, Florida and Mex and BCMNV isolate NL3.

Virus		TS		NL7		NY15		Florida		Mex	
		T[a]	N[b]	T	N	T	N	T	N	T	N
BCMV	TS										
	NL7	94	77								
	NY15	94	77	96	83						
	Florida	95	79	98	92	97	88				
	Mex	95	77	98	94	97	87	99	98		
BCMNV	NL3	82	46	83	58	83	58	83	54	83	58

[a] T = Total sequence homology of PCR product

[b] N = sequence homology of the N-terminal region of coat protein.

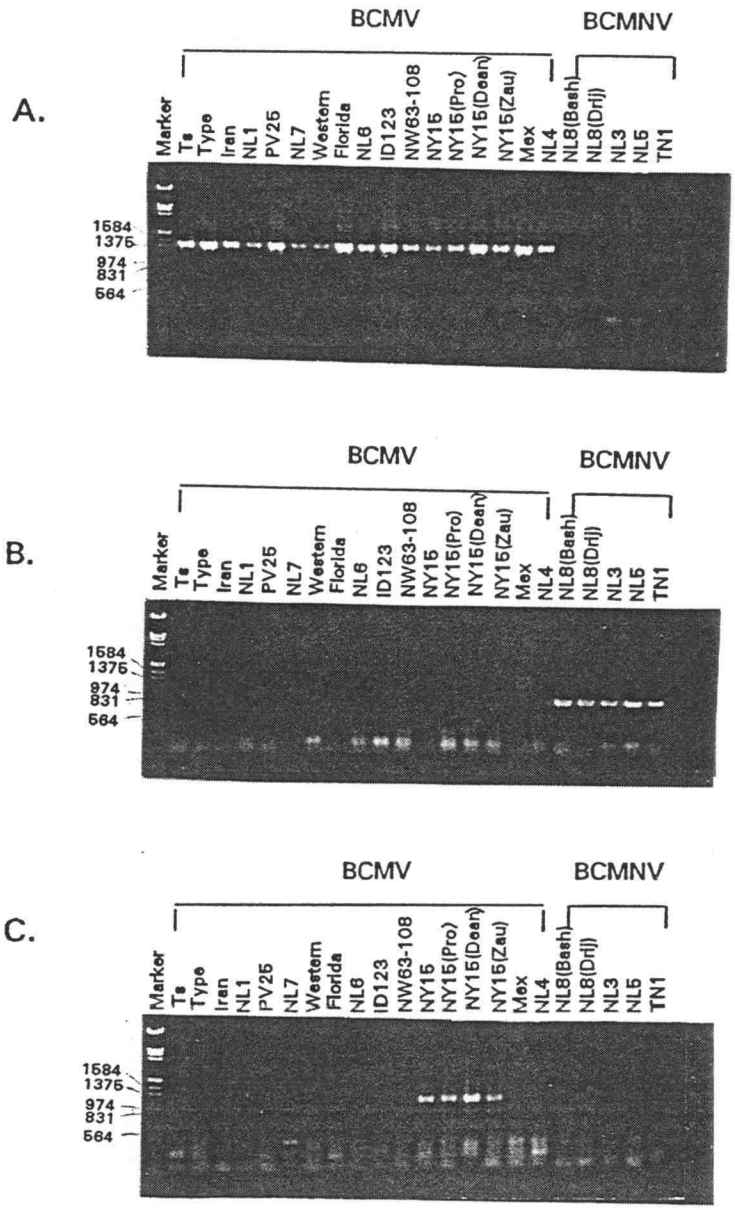


Figure 4 (A-C). Tests of RT-PCR amplification of 22 BCMV and BCMNV isolates with primer pair Dbcmv/Ubcmv(A); Dnl3/Unl3(B) and Dts/Uny15(C).

generated detectable PCR products from non-infected plant tissue nucleic acid extracts.

4.2.3 Sensitivity of RT-PCR and detection of mixed infection

When a dilution series of purified viral RNA of isolates TS, NY15 and NL3 was used for amplification by primer pairs Dbcmv/Ubcmv, Dts/Uny15 and Dnl3/Unl3 respectively, the minimum amounts of viral RNA required for detection were 10 pg for primer pairs Dbcmv/Ubcmv and Dnl3/Unl3 and 100 pg for primer pair Dts/Uny15.

Experimental detection of mixed viral infections was performed by RT-PCR using primer pairs of Dbcmv/Ubcmv and Dnl3/Unl3 either individually or mixed together. Each primer pair, when used individually, yielded a band of homologous viral cDNA from intentional mixtures of BCMV and BCMNV RNA. However, when the primer pairs were used together, only BCMNV cDNA was generated (i.e., no RT-PCR product of BCMV was produced).

4.2.4 Restriction endonuclease analysis

Results of restriction endonuclease digestion of BCMV and BCMNV RT-PCR products are summarized in Table 5 and 6. Restriction enzyme *XbaI* differentiated two BCMNV pathogroups: PG-III and PG-VI when applied to BCMNV PCR products (Figure 5). None of the four restriction enzymes generated restriction profiles that precisely differentiated the five

BCMV pathogroups (Figure 6 A-D), but some restriction enzymes were potentially useful. For example, *EcoRI* cut the RT-PCR products from all isolates of BCMV-PG-I and three isolates (NL6, ID123 and NW63-108) of PG-IV, but no other isolates. Thus *EcoRI* differentiated BCMV-PG-I from PG-II, V and VII. Similarly *BamHI* differentiated BCMV-PG-VII from PG-I,II, and V.

Table 5. Digestions of BCMV RT-PCR products by restriction endonucleases *XbaI*, *EcoRI*, *BamHI* and *EcoRV* (+ = cut; - = not cut)

isolates	pathogroup	*XbaI*	*EcoRI*	*BamHI*	*EcoRV*
Ts	I	+	+	-	-
Type	I	+	+	-	-
Iran	I	+	+	-	-
NL1	I	+	+	-	-
PV25	II	+	-	-	-
NL7	II	-	-	-	-
Florida	IV	-	-	+	+
Western	IV	-	-	-	-
NL6	IV	-	+	-	+
ID123	IV	+	+	-	-
NW63-108	IV	+	+	-	-
NY15	V	-	-	-	+
NY15(Prov)	V	-	-	-	+
NY15(Dean)	V	-	-	-	+
NY15(Zau)	V	-	-	-	+
Mex	VII	-	-	+	+
NL4	VII	-	-	+	+

Table 6. Digestions of BCMNV RT-PCR products by restriction endonucleases *XbaI*, *EcoRI*, *BamHI* and *EcoRV* (+ = cut; - = not cut)

strains	pathogroup	*XbaI*	*EcoRI*	*BamHI*	*EcoRV*
NL8(Bash)	III	-	+	-	-
NL8(Drij)	III	-	+	-	-
NL3	VI	+	+	-	-
NL5	VI	+	+	-	-
TN1	VI	+	+	-	-

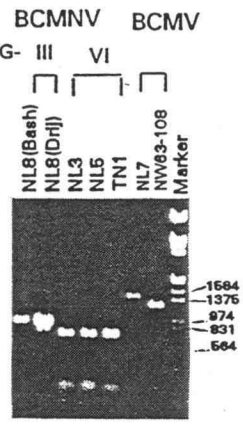


Figure 5. Restriction endonuclease XbaI digestion of RT-PCR products amplified from BCMNV isolates (left 5 lanes) and two BCMV isolates.

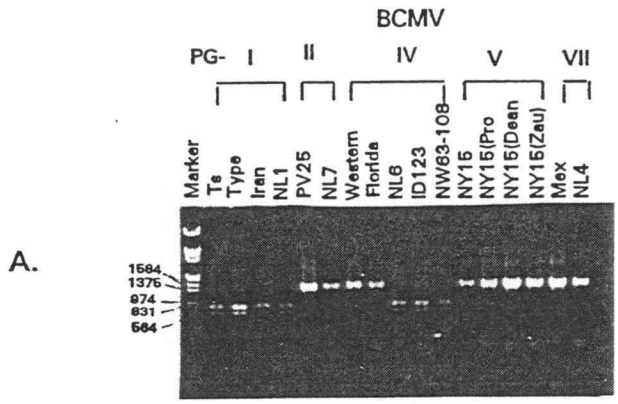
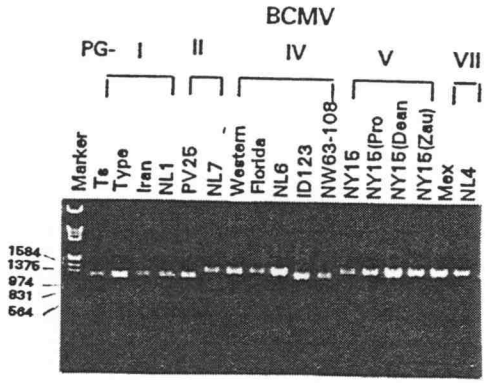
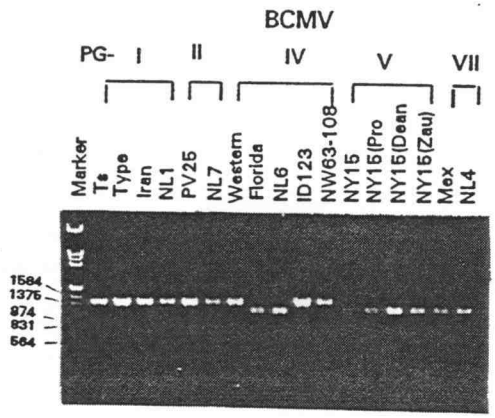


Figure 6. Restriction endonuclease digestion of RT-PCR products amplified from BCMV isolates by EcoRI(A), XbaI(B), EcoRV(C), and BamHI(D).

B.



C.



D.

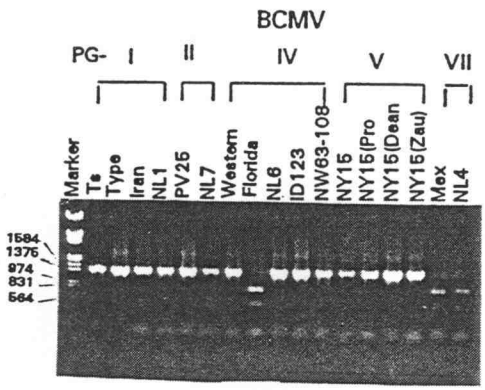


Figure 6. (continued)

5. CONCLUSION AND DISCUSSION

5.1 Polyclonal antibody serology

In contrast with a prior failure to distinguish BCMV and BCMNV with polyclonal antisera by DAS-ELISA (Lana et al., 1988) similar methodology in the present study provided clear distinction of these two viruses. This rather simple approach, in retrospect then, has accomplished virus-specific detection that is equivalent to antibodies to N-terminal amino-acid sequences (Khan et al., 1990) application of BCMV-incited monoclonal antibodies (Wang et al, 1984; Mink et al., 1992), and HPLC analysis of partially digested viral coat protein (McKern et al., 1992). Efficacious use of polyclonal antisera is more direct, less expensive, and more easily generated and shared. Use of polyclonal antisera is also more feasible and practical for moderately funded or equipped laboratories (i.e., more universally applicable).

Data from this study also indicated that the DAS-ELISA format was necessary for differentiating BCMV and BCMNV, in agreement with Koenig & Paul (1982). The two ELISA formats differ in two specific ways. In ACP-ELISA, the virus is bound directly to the microtiter plate and is exposed to high pH buffer that can cause capsid degradation of some viruses (Murphy and D'Arcy, 1991). In such cases, internal antigenic determinants less variable among potyviruses (Shukla et al., 1988), may bind polyclonal antibodies. In DAS-ELISA, plate-bound IgG molecules react with better-preserved antigens

(i.e., intact determinants) in pH-neutral buffer. Secondly, both the primary and secondary antibodies (i.e., specific IgG and the alkaline phosphatase-IgG conjugate) bind only to virus specific determinants (Clark & Adams, 1977). Thus, two specific antigen-antibody unions are inherent in the immunosorption "positive reaction".

TiterMax adjuvant (CytRx Corp., Norcross, GA) was used in this study, an adjuvant reported to comprise an emulsion of microparticles in water and oil and to induce high, and long-lasting antibody titers in experimental animals (Bennett et al, 1992). The anti-viral antibody titer observed in this study was at least comparable to the Freund's Complete Adjuvant used in prior studies of our laboratory. The unprecedented BCMV vs. BCMNV specificity of the two generated polyclonal antisera could be attributable to this adjuvant, perhaps in combination with a high degree of purity and integrity of the viral antigens.

Differences in ELISA reaction intensity within assay sets (e.g., Table 3) are typically the result of two factors: non-standardized antigen concentrations and distinct serological affinities. In this study, quantitative ELISA was not attempted; thus, test antigens varied quantitatively, consisting of desiccated leaf-tissue samples from bean cv. Red Kidney plants that had been inoculated, respectively, with 22 virus isolates. Likewise, the data from DAS- and ACP-ELISA are not comparable, because the two ELISA formats were not tested in the same experiment and antigen preparations were

not identical (i.e., were sampled and prepared separately) in the two assays. Thus, interpretations of ELISA data were based entirely upon a discernible "reaction" (i.e., A_{405}, 0.2 to 3.0) vs. "no reaction" (i.e., 0.00). For this purpose, calculations of variance or virus-detection thresholds (e.g., Hampton et al, 1992) were considered unnecessary.

5.2 Amino acid sequences

The N-terminal region of coat protein is generally recognized as being divergent among potyviruses (Shukla et al., 1988). This divergence, in fact, is a principal basis for the serological distinctions among potyviruses (Edwardson & Christie, 1980). More recently, the coat protein coding region and 3′-end untranslated leader has become recognized as the taxonomically significant portion of potyvirus genomes (Barnett, 1991).

N-terminal amino acid sequences differed in both length and diversity (i.e., 46 to 58% homologous) between the BCMV and BCMNV isolates investigated, and differed much less (i.e., 77 to 98% homologies) among isolates within BCMV, verifying the current taxonomic relationship between the two viruses and supporting the concept that BCMNV is a distinct entity, apart from BCMV. Even the minor sequence differences among BCMV isolates occurred in the N-terminal region. For example, isolate TS shared only 77 to 79 % putative amino acid sequence homology with isolates NY15, NL7, Florida and Mex (Table 4), as did NY15 (i.e., shared 83 to 88% homology with isolates

NL7, Florida and Mex). However, sequences of isolates NL7, Florida and Mex were uniquely conserved (i.e., 92 to 98% homology). Beyond their significance in virus/pathotype relationships, such sequence homology or diversity probably also affects virus functions in ways yet to be determined, perhaps even pathotype determination.

To date, neither potyviral nor BCMV nucleotide sequences associated with pathotypes have been identified. In selecting five BCMV isolates (i.e., pathogroup representatives) for examination of their genomic 3'-terminal regions, it was recognized that meaningful insights into pathotype determination were unlikely. Indeed, molecular studies of additional isolates per pathogroup, though beyond the scope of this study, are necessary. For this study, limited but reliable molecular approaches for pathogroup differentiation were explored, deferring to future studies for defining those genomic sequences determining BCMV pathogenicity. Only when such sequences are defined will it be possible to design truly pathogroup-specific molecular probes.

Khan et al.,(1992) reported 98% amino acid sequence homology between BCMV isolates NL1 and NY15 and noted that the aphid transmission motif DAG was absent in his NY15 isolate. This motif was also missing in the NY15 isolate of the present study.

5.3 RT-PCR based assay

The RT-PCR procedure developed herein provided specific

detection of BCMV, BCMNV and some of their variants. As illustrated in Figure 7, besides distinguishing the two viruses, the technique also detected one BCMV pathogroup (BCMV-PG-V), differentiated two BCMNV pathogroups (BCMNV-PG-III and PG-VI) and partially separated certain BCMV pathogroups (i.e. separated one pathogroup from two or three distinct pathogroups). Although only a few members (2-5 isolates) of each pathogroup were tested, the specificities obtained exceed any currently used serological or molecular methods and demonstrate the potential of this approach until complete sequences of BCMV and BCMNV are determined.

For the three primer pairs tested, their detection limits for purified RNA ranged from 100 to 10 pg, which were equal to 2 ng to 200 pg of purified virus. Detection sensitivities, then, approximated those of ELISA in detecting pea seed borne mosaic virus (Kohnen, 1992). When applied to nucleic acid extracts from infected plant tissues, all primer pair amplified sufficient PCR products to produce clear gel electrophoretic bands, even though quantitatively variable among isolates and samples. The total RNA required for one reaction was 1 ug, which was easily obtained from less than 0.05 g tissue. Some anomalous smears occurred in gels receiving RT-PCR products generated from pathogroup-specific primer pair Dts/Uny15 (Figure 4C). Such smears probably represented truncated sequences generated from non-viral nucleic acids and could be related to the smaller size (17

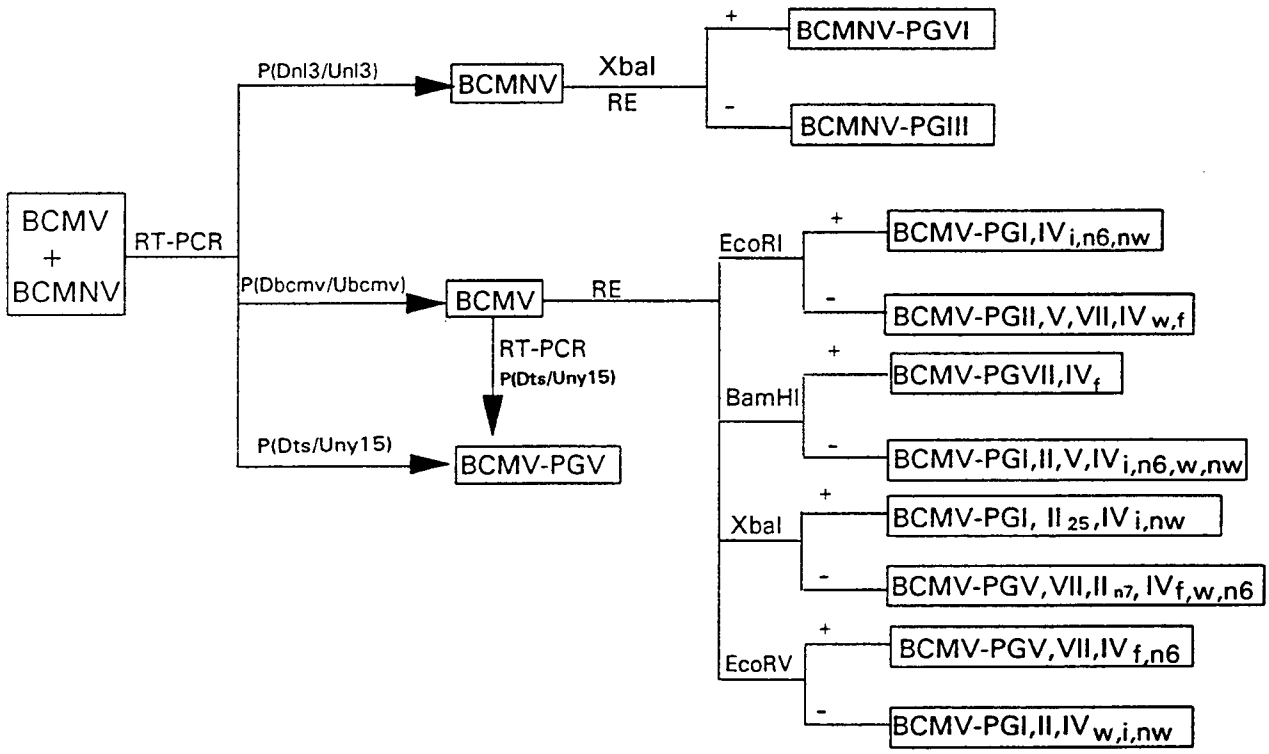


Figure 7. Schematic diagram for detection and differentiation of BCMV, BCMNV and their pathogroups by RT-PCR and restriction endonuclease analysis. P = primer; RE = restriction endonuclease digestion; + = cut by restriction; - = not cut by restriction enzyme; PGI to PGVII = pathogroup I to VII. The subscripts followed in some pathogroups indicate isolates in that group. i = ID123; n6 = NL6; nw = NW63-108; f = Florida; w = Western; n7 = NL7; 25 = PV25.

nucleotides) of the Uny15 primer (i.e., other primer pairs comprised greater than 20 nucleotides).

Detection of mixed virus infections with two primer pairs in the same tube proved unsuccessful, in which case only one RT-PCR product was amplified to a lesser amount than when alone. In this case, there is probably both competition for nucleotides in the reaction mixture, but also cross-interference in template and product assemblies.

This RT-PCR procedure was based on only a portion (about one fifth) of BCMV and BCMNV genome information. With such limitation, the methodology demonstrates great potential in the application of specific detection. With increasing sequence information, additional pathogroup-specific primers may be more readily developed. Results from this study demonstrate that RT-PCR detection of a single nucleotide difference is both theoretically possible and practically feasible (e.g., Dts/Uny15). Thus, this methodology provides an almost unlimited potential for detection of subtle molecular variants.

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APPENDICES

APPENDIX 1

Virus Purification

Protocol by Karen E. Keller

Buffers

1. Extraction buffer (pH 7.5) (1 litter)

Na_2SO_3	1.0 g
EDTA	3.7 g
K_2HSO_4	70.5 g
KH_2SO_4	12.9 g

Dissolve above chemicals in double distilled water, final volume 1 liter ,adjust pH to 7.5.

2. .02 M potassium phosphate buffer (pH 8.2) (1 liter)

K_2HSO_4	3.3 g
KH_2SO_4	.14 g
2-mercaptoethanol	1 ml

Dissolve above chemical in double distilled water, bring volume to 1 liter and adjust pH to 8.2.

3. .6 M NaCl buffer (50 ml)

Dissolve 1.75 g of NaCl in 50 ml of .02 M potassium phosphate buffer (pH 8.2)

Protocol

1. Add 100 g of well chilled tissue to 200 ml of .5 M extraction buffer and 50 ml each carbon tetrachloride and chloroform and blend until well homogenized.

2. Centrifuge at 8,000 RPM for 15 min.

3. Filter the supernatant through glass wool into a graduated cylinder. Measure volume and add PEG 8000 at 4% wt/vol. Stir slowly at 4 C for 1 hour

4. Spin at 8,000 RPM for 15 min.

5. Resuspend pellet in 40 ml of .02 M phosphate buffer and stir slowly at 4 C for 1 hour. Pour sample into SW40 ultra clear centrifuge tubes, 10 ml per tube.

6. Add 4.75 g of CsCl to each tube and dissolve. Well balance each tube and centrifuge at 25,000 RPM for 20 hours.

7. Remove viral band with a syringe. Precipitate virus from the CsCl by adding .6 M NaCl buffer at 1:1 ratio, Add PEG 8,000 at 5% wt/vol and centrifuge at 9,000 RPM for 10 min.

8. Resuspend viral pellet in 500 ul of .02 M potassium phosphate buffer. Measure viral concentration by taking spectrophotometer reading.

APPENDIX 2

DAS-ELISA and ACP-ELISA protocol

from laboratory of R.O. Hampton

Buffers

1. Coating buffer (1 liter)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	.20 g

Add 990 ml of dd H_2O. Adjust pH to 9.6. Bring volume to 1 liter. Store at 4 C.

2. 5X PBS (2 liter)

NaCl	80.0 g
KH_2PO_4	2.0 g
Na_2HPO_4	11.5 g
K2Cl	2.0 g
NaN_3	2.0 g

Mix ingredients in 1900 ml dd H_2O. Adjust pH to 7.4. Bring the volume to 2 liter. Store at room temperature.

3. Washing buffer (1 liter)

5X PBS	200 ml
dd H_2O	800 ml
Tween 20	1 ml

Mix together and store at room temperature.

4. Virus buffer (1 liter)

5X PBS	200 ml
polyvinylpyrrolidone	20 g
ovalbumin	2 g
Tween 20	1 ml

Mix ingredients in dd H_2O. Adjust pH to 7.4 and bring volume to 1 liter. Store at 4 C.

5. Substrate Buffer (1 liter)

Diethenolamine	97 ml
NaN_3	.2 g

Dissolve ingredients in dd H_2O. Adjust pH to 9.8 and bring volume to 1 liter. Store at 4 C.

6. Antigen buffer (1 liter)

5X PBS	200 ml
dd H_2O	800 ml
NaDIECA	2.25 g

Mix and store at room temperature.

DAS-ELISA Protocol

1. Make specified dilution of IgG in coating buffer. Mix well and add 200 ul to each well.
2. Incubate at 37 C for 4 hour or at 4 C overnight.
3. Wash plate 3 times, 3 min each time with washing buffer.
4. Grind plant sample with virus buffer and add 200 ul plant sap to each well.
5. Repeat step 2 and 3.
6. Dilute virus specific conjugate to specific concentration with virus buffer and add 200 ul to each well.
7. Repeat step 2 and 3.
8. Dissolve substrate tablets (Sigma 104 Phosphatase substrate tablet) in substrate buffer at concentration of 1 mg/3 ml and add 200 ul to each well.
9. Take A_{405} read upon color developed.

ACP-ELISA protocol

1. Grind sample in antigen buffer and add 200 ul to each well.
2. Incubate plate at 37 C for 2 hour or at 4 C overnight.
3. Wash plates 3 times, 3 min each time with washing buffer.

4. Make specific dilution of antiserum and add 200 ul to each well(if the antiserum cross react with healthy plant tissue, cross absorb the antiserum with 1:50 health plant sap one hour before antiserum is to be added).

5. Repeat step 2 and 3.

6. Mix specified amount of goat-rabbit alkaline phosphatase conjugate with virus buffer and add to plate.

7. Repeat step 2 and 3.

8. Add 200 ul substrate solution (concentration of 1 mg/3 ml) to each well.

9. Take reading at A_{405} upon color developed.