Infectivity analysis of two variable DNA B components of *Mungbean yellow mosaic virus-Vigna* in *Vigna mungo* and *Vigna radiata*

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Mungbean yellow mosaic virus-Vigna (MYMV-Vig), a *Begomovirus* that causes yellow mosaic disease, was cloned from field-infected blackgram (*Vigna mungo*). One DNA A clone (KA30) and five different DNA B clones (KA21, KA22, KA27, KA28 and KA34) were obtained. The sequence identity in the 150-nt common region (CR) between DNA A and DNA B was highest (95%) for KA22 DNA B and lowest (85·6%) for KA27 DNA B. The Rep-binding domain had three complete 11-nt (5′-TGTATCGGTGT-3′) iterons in KA22 DNA B (and KA21, KA28 and KA34), while the first iteron in KA27 DNA B (5′-ATCGGTGT-3′) had a 3-nt deletion. KA27 DNA B, which exhibited 93·9% CR sequence identity to the mungbean-infecting MYMV, also shared the 3-nt deletion in the first iteron besides having an 18-nt insertion between the third iteron and the conserved nonanucleotide. MYMV was found to be closely related to KA27 DNA B in amino acid sequence identity of BV1 (94·1%) and BC1 (97·6%) proteins and in the organization of nuclear localization signal (NLS), nuclear export signal (NES) and phosphorylation sites. Agroinoculation of blackgram (*V. mungo*) and mungbean (*V. radiata*) with partial dimers of KA27 and KA22 DNA Bs along with DNA A caused distinctly different symptoms. KA22 DNA B caused more intense yellow mosaic symptoms with high viral DNA titre in blackgram. In contrast, KA27 DNA B caused more intense yellow mosaic symptoms with high viral DNA titre in mungbean. Thus, DNA B of MYMV-Vig is an important determinant of host-range between *V. mungo* and *V. radiata*.

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1. Introduction

Yellow mosaic disease (YMD), which affects five important pulse crops, blackgram, mungbean, French bean, pigeon pea and soybean, causes an annual yield loss of about $ 300 million (Varma *et al* 1992). *Vigna mungo* (blackgram) is the third major pulse crop cultivated in the Indian sub-continent. It is highly prone to YMD (Nene 1973) which is a widespread problem because all cultivated varieties are susceptible to the disease. YMD in *V. mungo* and

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Abbreviations used: CR, Common region; ds, double-stranded; kb, kilobase pair(s); kDa, kiloDalton(s); lin, linear; MYMV-Vig, *Mungbean yellow mosaic virus-Vigna*; NES, nuclear export signal; NLS, nuclear localization signal; nt, nucleotide; oc, open circular; OD, optical density; ORF, open reading frame; Rep, replication-associated protein; RF, replicative form; sc, supercoiled; ss, single-stranded; YMD, yellow mosaic disease.

V. radiata (mungbean) was first reported by Nariani (1960). It causes 85–100% yield loss when the plants are infected at the seedling stage (Nene 1973). The disease is caused by begomoviruses, which have been shown to have bipartite genomes (Honda and Ikegami 1986; Vanitharani *et al* 1996; Mandal *et al* 1997; Karthikeyan *et al* 2004). Various isolates of the begomoviruses causing YMD have been placed in two virus species, *Mungbean yellow mosaic India virus* (MYMIV) and *Mungbean yellow mosaic virus* (MYMV) on the basis of nucleotide sequence identity (Fauquet *et al* 2003). The blackgram-infecting isolate cloned by us (Vanitharani *et al* 1996; Pooggin *et al* 2003) has been named by Fauquet *et al* (2003) as *Mungbean yellow mosaic virus-Vigna* (MYMV-Vig). Agroinfection with infectious clones of DNA A and DNA B has confirmed the bipartite nature of MYMV (Mandal *et al* 1997) and MYMV-Vig (Jacob *et al* 2003; Karthikeyan *et al* 2004). This paper reports the analysis of two variable DNA B components cloned from field-infected *V. mungo* plants. On the basis of sequence identity analysis and symptom development in agroinoculated *V. mungo* and *V. radiata* plants, the two DNA B components were found to be distinctly different.

2. Materials and methods

2.1 *Source of infected sample and seeds*

Infected *V. mungo* (L.) Hepper leaves exhibiting yellow mosaic symptoms were collected from the experimental field of the National Pulses Research Centre, Vamban, Tamil Nadu. Seeds of *V. mungo* cv. CO-5 and *V. radiata* cv. CO-4 were obtained from the Tamil Nadu Agricultural University, Coimbatore.

2.2 *Southern hybridization*

Total DNA from agroinoculated plants was extracted using CTAB (Porebski *et al* 1997) and DNA concentration was estimated in a fluorometer using the Hoechst dye 33258. A range of DNA samples (0·008, 0·04, 0·2 and 1 μg) as 5-fold dilutions were separated in 0·8% agarose gels in 1 X TNE buffer (Hong and Stanley 1996). After ethidium bromide staining, DNA was alkali-denatured and transferred (Southern 1975) to Zeta-probe nylon membrane (Bio-Rad Laboratories, USA). Southern transfer was also done under non-denaturation conditions (Veluthambi *et al* 1988) to identify ssDNA of the virus. DNA probes were prepared using Megaprime™ DNA labelling system (Amersham International Plc. Ltd, UK) and [α-^{32}P]dCTP (~ 3000 Ci/mmol, BRIT, Mumbai). Hybridization was carried out overnight at 65°C in a hybridization oven. Post-hybridization washes were done at high-stringency (three washes, sequentially with 2X SSC/0·1% SDS, 0·5X SSC/0·1% SDS and 0·1X SSC/0·1% SDS at 65°C). Konica X-ray films (type AX) were used for autoradiography.

2.3 *Sequence analysis*

Nucleotide and amino acid data analysis were carried out using the GCG package (University of Wisconsin, USA) (Devereux *et al* 1984). The geminivirus sequences which were accessed from the EMBL and GenBank databases and used for comparisons are listed in table 1. CLUSTAL W (Thompson *et al* 1994) was used for multiple alignment of sequences. Nuclear localization signal (NLS) and nuclear export signal (NES) were identified in the BV1 protein of MYMV-Vig DNA Bs using sequence analysis programs like BLAST, FASTA, SSEARCH and HMMER. Pattern/peptide match program in PIR website was used to identify putative phosphorylation sites in MYMV-Vig DNA Bs.

Table 1. Accession numbers of DNA B sequences.

Virus	Accession number
ACMV-[KE]	J02058
ACMV-[NG]	X17096
ICMV	Z24759
MYMIV	AF142440
MYMIV-[Cp]	AF503580
MYMIV-[Sb]	AY049771
MYMIV-[SbTN]	AJ420331
MYMV	D14704
MYMV-Vig	
*KA21	AJ439059
*KA22	AJ132574
*KA27	AF262064
*KA28	AJ439058
*KA34	AJ439057
SACMV	AF155807
SLCMV-[Col]	AJ314738
ToLCNDV-Svr	U15017

African cassava mosaic virus: Kenya ACMV-[KE], Nigeria ACMV-[NG]; *Indian cassava mosaic virus* ICMV; *Mungbean yellow mosaic India virus* MYMIV: Cowpea isolate MYMIV-[Cp], Soybean isolate MYMIV-[Sb], MYMIV-[SbTN]; *Mungbean yellow mosaic virus* MYMV; *South African cassava mosaic virus* SACMV; *Sri Lankan cassava mosaic virus* SLCMV-[Col]; *Tomato leaf curl New Delhi virus*: Severe strain ToLCNDV-Svr.
Accession number of DNA A of MYMV-Vig: AJ132575.
*KA21, KA22, KA27, KA28 and KA34 are clones of five different DNA B components of MYMV-Vig. These sequences were determined as a part of this study.

2.4 *Agroinfection*

Partial dimer clones of the DNA A (pGA1·9A), KA22 (pGA1·9B22) (Jacob *et al* 2003) and KA27 (pGA1·5B27) DNA B components of MYMV-Vig, each containing two origins of replication as direct repeats, were constructed in a modified version of the binary vector pGA472 (An *et al* 1985). The partial dimer clone pGA1·5B27 was constructed in this study by cloning the 2·67-kb full-length *Cla*I fragment of pKA27 and its 1·4 kb *Cla*I/*Hind*III fragment (0·5 mer) to make a partial tandem repeat. The partial dimer clones of KA22 (pGA1·9B22) and KA27 (pGA1·5B27) DNA Bs were mobilized from *Escherichia coli* into *Agrobacterium tumefaciens* strain C58 (Sciaky *et al* 1978) by triparental mating (Ditta *et al* 1980). The clone pGA1·9A was transformed by electroporation (Bilang *et al* 1994) into *A. tumefaciens* strain C58. *Agrobacterium* transconjugants and transformants with partial dimer clones were confirmed by Southern analysis (data not shown).

Agroinoculation was performed according to Mandal *et al* (1997) with a few modifications (Jacob *et al* 2003). *A. tumefaciens* C58 strains, one harbouring the partial dimer clone of DNA A (pGA1·9A) and the second with the partial dimer clone of DNA B (pGA1·9B22 or pGA1·5B27), were grown in AB minimal medium (Chilton *et al* 1974) (pH 7·0) to 1·0 absorbance at 600 nm in a shaker at 28°C. The cells were harvested by centrifugation at 1000 *g* for 10 min at 28°C and the pellet was resuspended in an equal volume of AB minimal medium (pH 5·6) containing 100 μM acetosyringone (Aldrich Chemical Company, USA). *V. mungo* cv. CO-5 and *V. radiata* cv. CO-4 seedlings were prepared as described by Mandal *et al* (1997). Seeds were surface-sterilized and held overnight in dark in a Petri dish with a wet Whatman No.1 circle at 25°C ± 2°C. Seed coat was removed and the hypocotyl region was pricked thrice with a sterile 30 G hypodermic needle. The seeds were immediately immersed in the *A. tumefaciens* culture. Infection was carried out overnight at 25°C ± 2°C, seeds were washed twice with sterile distilled water and sown in pots containing autoclaved potting mix (vermiculite/sand, 1 : 1). The germinated, agroinoculated seedlings were maintained in an illuminated growth chamber set at 25°C, with 60% relative humidity and 16/8 h light/dark regimen. Plants were watered regularly and nourished with Hoagland's solution twice a week. After a period of three weeks, plants were transferred from growth chamber to the greenhouse. Symptoms were monitored periodically and young trifoliate leaves exhibiting yellow mosaic symptom were harvested and stored at – 70°C for viral DNA analysis. Total DNA from the trifoliate leaves pooled from five to ten mock-inoculated and agroinoculated *V. mungo* and *V. radiata* plants was extracted as described earlier (Porebski *et al* 1997).

3. Results

3.1 *Genome organization of MYMV-Vig*

The genome organization of MYMV-Vig, deduced from the nucleotide sequences, resembles that of the Old World begomoviruses (figure 1). All five DNA B components (KA21, KA22, KA27, KA28 and KA34) had two predicted open reading frames (ORFs) (BV1 and BC1). All deduced ORFs had the potential to code for proteins larger than 10 kDa. As in other bipartite begomoviruse (Hanley-Bowdoin *et al* 1999), DNA A and DNA B of MYMV-Vig shared a common region (CR) of 150-nt (figure 1).

3.2 *Nucleotide sequence identity in the CR of MYMV-Vig genomic components*

The CR, which carries the origin of replication, exhibits high sequence identity between the cognate DNA A and DNA B of a given bipartite geminivirus (Lazarowitz 1992). The length of CR sequences in MYMV-Vig DNA A and DNA Bs was determined as 150-nt based on the nucleotide sequence homology. The CR contains a putative stem-loop structure with the conserved nonanucleotide sequence (5′-TAATATTAC-3′) in the loop (Lazarowitz 1992). This motif contains the nicking site for the initiation of rolling circle replication. The CR sequence identity between DNA A and DNA Bs of MYMV-Vig is shown in table 2. KA22 DNA B showed the highest CR sequence identity of 95% to that of DNA A, while KA27 DNA B shared the lowest CR sequence identity of 85·6%. KA27 DNA B showed the lowest total nucleotide sequence identity (72%) to KA22 DNA B. Positions of several restriction sites varied between KA22 and KA27 DNA Bs (figure 1). KA21, KA28 and KA34 DNA Bs shared a total nucleotide sequence identity of 96·9–99% with KA22 DNA B (results not shown).

Multiple alignment of DNA B components in the CR is shown in figure 2. Iterative sequence elements (iterons) (Arguello-Astorga *et al* 1994a,b) that have been demonstrated to constitute the Rep protein binding site (Eagle *et al* 1994; Fontes *et al* 1994a,b) were found in the CR region upstream of the stem-loop structure in all five DNA Bs of MYMV-Vig. Four DNA Bs (KA21, KA22, KA28 and KA34) of MYMV-Vig exhibited three 11-nt (5′-TGTATCGGTGT-3′) iterons in which TGT overlaps between the second and third iterons. A similar iteron organization was found in DNA A of MYMV-Vig (data not shown). KA27 DNA B has the complete overlapping second and third 11-nt iterons. However, the first iteron is only 8-nt long (5′-ATCGGTGT-3′) and lacks 3-nt (TGT) at the 5′ end. Besides, KA27 DNA B also carries an additional 18-nt sequence located between the third iteron and the stem-loop

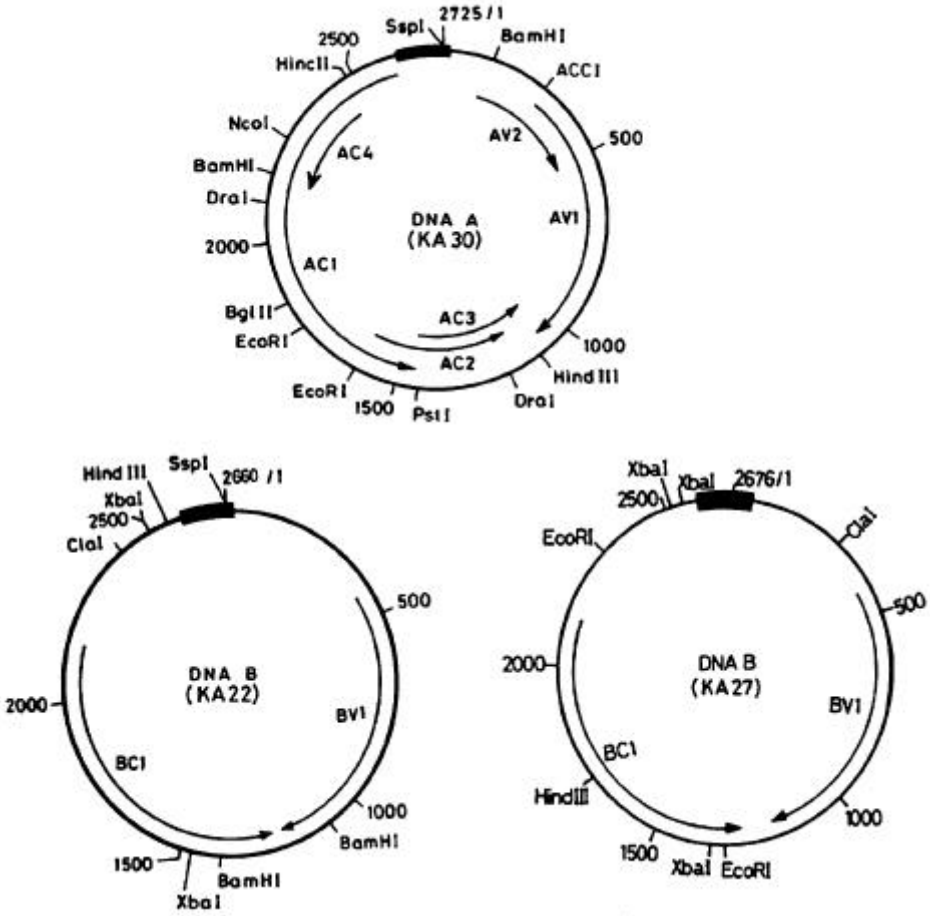

Figure 1. Genome organization of DNA A (KA30, 2725-nt) and DNA B (KA22, 2660-nt, KA27, 2676-nt) components of MYMV-Vig. All ORFs starting with the ATG codon and encoding proteins larger than 10 kDa are shown as solid arrows. The ORFs of virion-sense strands are designated as AV1, AV2 (DNA A) and BV1 (DNA B). ORFs on complementary-sense strands are designated as AC1, AC2, AC3 and AC4 (DNA A) and BC1 (DNA B). Filled box (■) represents the 150-nt CR for both components.

Table 2. Common region (CR) sequence identity (%) between DNA A and DNA B of mungbean yellow mosaic viruses.

Virus	CR sequence identity*
MYMV-Vig	
DNA A/KA21	86·3
DNA A/KA22	95·0
DNA A/KA27	85·6
DNA A/KA28	86·9
DNA A/KA34	94·6
MYMIV	83·5
MYMV	83·3

*The length of the CR taken for comparison is 150-nt [125-nt upstream and 25-nt downstream from the eighth nt (A) of the nonanucleotide sequence, 5′-TAATATTAC-3′].
KA21, KA22, KA27, KA28 and KA34 are five different DNA B components of MYMV-Vig.

structure. Interestingly, the 3-nt deletion in the first iteron and the 18-nt additional sequence after the third iteron were also found in the CR of mungbean-infecting MYMV DNA B reported from Thailand (Morinaga *et al* 1993). In addition, the CRs of KA27 and MYMV DNA B were 93·9% identical.

3.3 *Similarities of MYMV-Vig DNA B proteins and their motifs*

The deduced protein sequences from the two ORFs (BV1 and BC1) of DNA B components of MYMV-Vig were compared with those of other bipartite begomoviruses from the Old World. The percentage amino acid sequence identities obtained in these comparisons are presented in table 3. The BV1 protein of KA22 DNA B showed the highest amino acid sequence identity to MYMIV-[Cp] and

MYMIV-[Sb]. KA22 DNA B BC1 protein exhibited the highest amino acid sequence identity of 97·6% to MYMIV-[SbTN]. Interestingly, KA27 DNA B of MYMV-Vig showed the highest identity to MYMV (Morinaga *et al* 1993) in both BV1 (94·1%) and BC1 (97·6%) proteins. On the other hand, KA27 DNA B exhibited only 81·1–82·4% (BV1) and 91·9–92·3% (BC1) sequence identity in other four DNA Bs of MYMV-Vig (data not shown).

Multiple alignment of amino acid sequences of BV1 and BC1 proteins of MYMV-Vig DNA Bs with those of MYMIV and MYMV are shown in figures 3 and 4, respectively. In both BV1 and BC1, KA27 DNA B differed significantly from the other four DNA B components (KA21, KA22, KA28 and KA34) of MYMV-Vig. The amino acid differences in BV1 and BC1 proteins are discussed taking KA22 DNA B as the representative type. KA27 DNA B differs from KA22 DNA B in the positions of 63 and 35 amino acids in BV1 and BC1 proteins, respectively. Interestingly, in the 53 amino acid positions of BV1 (figure 3) and 29 amino acid positions of BC1 (figure 4) at which KA27 DNA B differed from KA22 DNA B, there was a perfect match between KA27 DNA B and MYMV DNA B (Morinaga *et al* 1993) at the corresponding amino acid positions.

Multiple alignment analysis showed that KA27 DNA B differed from other MYMV-Vig DNA Bs in many amino acid positions in both BV1 and BC1 proteins. An analysis was done to find out whether these changes in amino acid positions map to functional domains. A VirD2 type nuclear localization signal (NLS) (Tinland *et al* 1992) was identified in the N-terminus of BV1 protein (coordinates 26–41) of all the five DNA Bs (figure 5). Seven amino acid residues (PRRRHRK) were identical between the NLS of native VirD2 (*Agrobacterium*) and four DNA Bs (KA21, KA22, KA28 and KA34). However, only five amino acids in the NLS of KA27 and MYMV DNA Bs matched with that of VirD2 NLS (figure 5). A LxxxL motif (LYGPL, coordinates 189–193) (Ward and Lazarowitz 1999) found in the C-terminus of BV1 protein of four DNA Bs (KA21, KA22, KA28 and KA34) of MYMV-Vig is the proposed nuclear export signal (NES). However, in KA27 and MYMV DNA Bs the NES is composed of amino acids IYAPL.

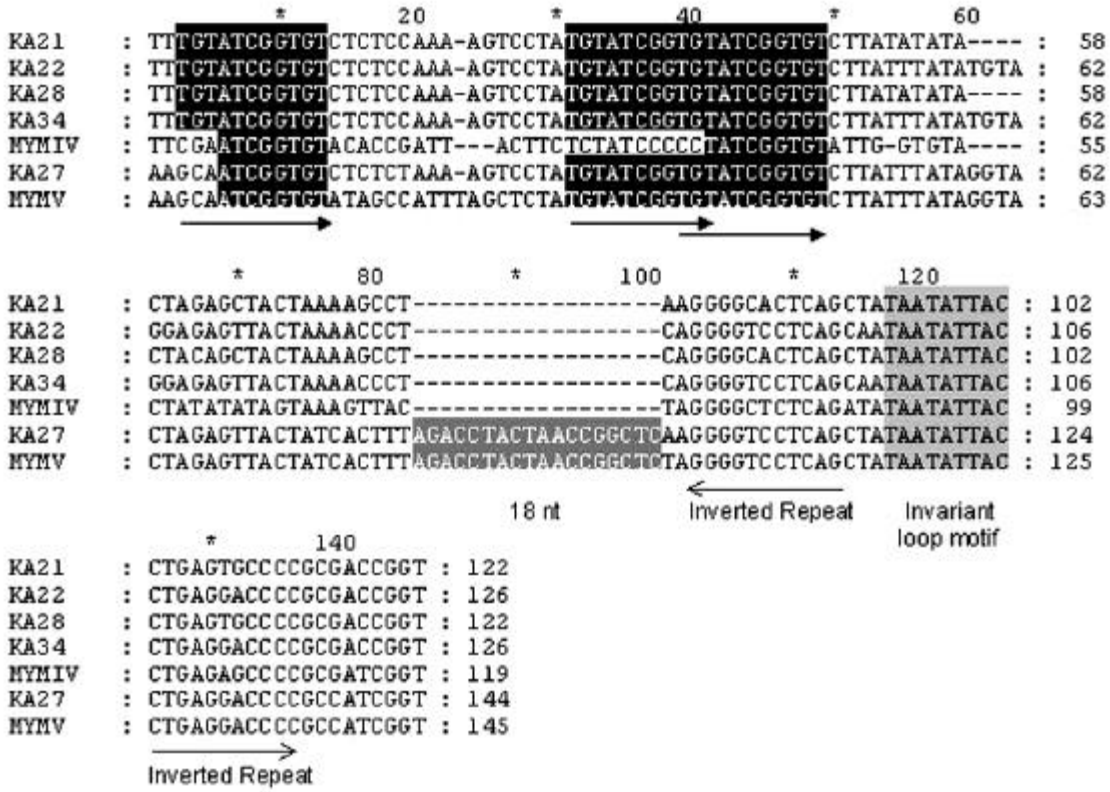

Figure 2. Alignment of the CR sequences of five DNA Bs (KA21, KA22, KA27, KA28 and KA34) of MYMV-Vig with those of MYMV and MYMIV. Gaps were inserted to provide maximum identity among the viruses. Iterative sequence elements (iterons) are shown as black boxes. The direction of iterons is indicated by arrows with filled head (➔). The nonanucleotide sequence (5′-TAATATTAC-3′) present in the loop region of the stem-loop structure is shown as a grey box. An 18-nt additional sequence present in KA27 DNA B and MYMV DNA B is highlighted. Inverted repeats capable of forming the stem-loop structure are indicated.

Therefore, KA27 and other four DNA Bs of MYMV-Vig differ by two amino acids in the NES. Putative phosphorylation sites were also identified in all the DNA Bs of MYMV-Vig. Phosphorylation in BV1 and BC1 is very important for protein-protein interaction (Sanderfoot *et al* 1996). Serine phosphorylation sites (RKLS, coordinates 40–43; and SRR, coordinates 200–202) identified in BV1 of KA21, KA22, KA28 and KA34 DNA Bs were absent in KA27 and MYMV DNA Bs. However, three additional phosphorylation sites (SQR, coordinates 198–200; TER, coordinates 227–229; and SSLD, coordinates 254–257) were identified in the BC1 of KA27 DNA B. TER and SSLD sites were common between KA27 and MYMV DNA Bs. Therefore, KA27 DNA B is closely related to MYMV DNA B and differs significantly from the other four DNA Bs of MYMV-Vig in the motifs for NLS, NES and protein phosphorylation.

3.4 *Comparison of infectivity of KA22 and KA27 DNA B components of MYMV-Vig in* V. mungo *and* V. radiata

The KA27 DNA B component of MYMV-Vig was strikingly similar to the DNA B of MYMV (a mungbean isolate from Thailand) (Morinaga *et al* 1993) on the basis of overall nucleotide sequence identity, CR organization, BV1 and BC1 amino acid sequences and in NLS and NES motifs. These observations raised an interesting question whether KA27 is a DNA B component of a mungbean-infecting virus. Therefore, the infectivity of KA27 and KA22 DNA Bs in *V. radiata* (mungbean) and *V. mungo* (blackgram) was compared by agroinoculation. Partial dimers of the DNA A (pGA1·9A) and two DNA Bs (KA22-pGA1·9B22, KA27-pGA1·5B27) of MYMV-Vig were used for agroinoculation of germinating *V. mungo* seeds (Mandal *et al* 1997; Jacob *et al* 2003).

Typical yellow mosaic symptoms (figure 6A) were induced in the trifoliate leaves of *V. mungo* upon agroinoculation with *A. tumefaciens* strains harbouring partial dimers of DNA A and KA22 DNA B. However, in *V. radiata* KA22 DNA B induced mild yellow mosaic symptoms (figure 6B).

A contrasting pattern of symptoms was observed with KA27 DNA B. Agroinoculation of *V. mungo* with DNA A and KA27 DNA B partial dimer clones produced mild yellow mosaic symptoms in the trifoliate leaves (figure 6A). In contrast, KA27 DNA B induced typical yellow mosaic symptoms in *V. radiata* (figure 6B). The experiment was performed thrice and the results were comparable. *V. mungo* and *V. radiata* plants agroinoculated with the partial dimer of DNA A alone or DNA B alone (KA22 or KA27) did not develop yellow mosaic symptoms (results not shown). The two DNA Bs (KA22 and KA27) are clearly differentiated on the basis of symptoms caused in *V. mungo* and in *V. radiata*. Whitefly transmission of KA22 and KA27 components in *V. mungo* and *V. radiata* has not been studied yet.

3.5 *Comparison of viral titre in* V. mungo *and* V. radiata *agroinoculated with KA22 and KA27 DNA B components of MYMV-Vig*

We evaluated whether the difference in the intensity of yellow mosaic symptoms caused by KA22 and KA27 DNA Bs of MYMV-Vig in *V. mungo* (KA22 DNA B-typical yellow mosaic symptom and KA27 DNA B-mild yellow mosaic symptom) and *V. radiata* (KA22 DNA B-mild yellow mosaic symptom and KA27 DNA B-typical yellow mosaic symptom) is reflected in the titre of the viral DNA.

Table 3. Amino acid sequence identities (%) between MYMV-Vig DNA Bs and other Old World bipartite begomoviruses.

	MYMV-Vig BV1					MYMV-Vig BC1				
Virus	KA21	KA22	KA27	KA28	KA34	KA21	KA22	KA27	KA28	KA34
ACMV-[KE]	52·1	52·1	53·3	52·5	51·7	71·6	71·6	71·2	71·6	71·6
ACMV-[NG]	55·7	55·7	55·7	55·7	55·3	72·2	72·2	71·6	72·2	72·2
ICMV	38·2	38·2	39·2	39·0	37·5	52·7	52·4	51·0	52·4	52·4
MYMIV	95·2	95·2	80·3	94·5	94·5	95·3	95·6	89·6	95·6	95·3
MYMIV-[Cp]	**97·6**	**97·6**	82·4	**96·8**	**96·8**	96·6	96·9	91·6	96·9	96·6
MYMIV-[Sb]	**97·6**	**97·6**	82·4	**96·8**	**96·8**	95·9	96·3	91·6	96·3	95·9
MYMIV-[SbTN]	96·4	96·4	82·1	95·7	95·7	**97·3**	**97·6**	91·3	**97·6**	**97·3**
MYMV	80·5	80·5	**94·1**	80·5	79·7	91·9	91·9	**97·6**	91·9	91·6
SACMV	50·3	50·3	51·7	50·3	50·0	66·1	68·1	67·1	68·1	68·1
SLCMV-[Col]	38·6	38·6	39·2	38·6	37·8	54·8	54·8	53·4	54·8	54·8
ToLCNDV-Svr	37·9	37·9	37·8	37·9	37·5	45·8	45·8	47·7	45·8	45·8

Bold types indicate the highest score for each comparison.

Total DNA was extracted from upper trifoliate leaves of agroinoculated *V. mungo* and *V. radiata* plants and subjected to Southern hybridization analysis. The DNA A and DNA B probes used were devoid of CR to analyse DNA A or DNA B molecules specifically. DNA A-specific signals are shown in figure 7. Virus-specific signals were not observed in mock-inoculated *V. mungo* and *V. radiata* plants. Two major bands moving at ~ 2·7 kb and ~ 1·8 kb regions lighted up upon agroinoculation with DNA A and DNA B partial dimers. Under non-denaturation blotting conditions (Veluthambi *et al* 1988) only ~ 1·8 kb band hybridized to the probe suggesting the presence of single-stranded (ss) virion DNA (data not shown). S1 nuclease treatment revealed the presence of a small amount of supercoiled (sc) dsRF DNA at the 1·8 kb position. The band moving at ~ 2·7 kb corresponds to open circular (oc) and linear (lin) dsRF DNA.

The intensity of signals (primarily at 1·8 kb position) was compared in adjacent lanes which had 5-fold difference in the amount of total DNA. In *V. mungo*, the DNA A signal in 0·2 μg of KA22-infected plants is about 10-times more intense than the corresponding sample in KA27-infected plants (figure 7). A contrasting picture is seen in *V. radiata*. The DNA A signal in 0·2 μg of KA27-infected plants is about 10-times more intense than the corresponding sample of KA22-infected *V. radiata* plants. Thus, viral DNA A titre is higher in *V. mungo* than in *V. radiata*, upon agroinoculation with KA22 DNA B. However, DNA A titre in *V. radiata* is higher than in *V. mungo* when agroinoculation involved KA27 DNA B.

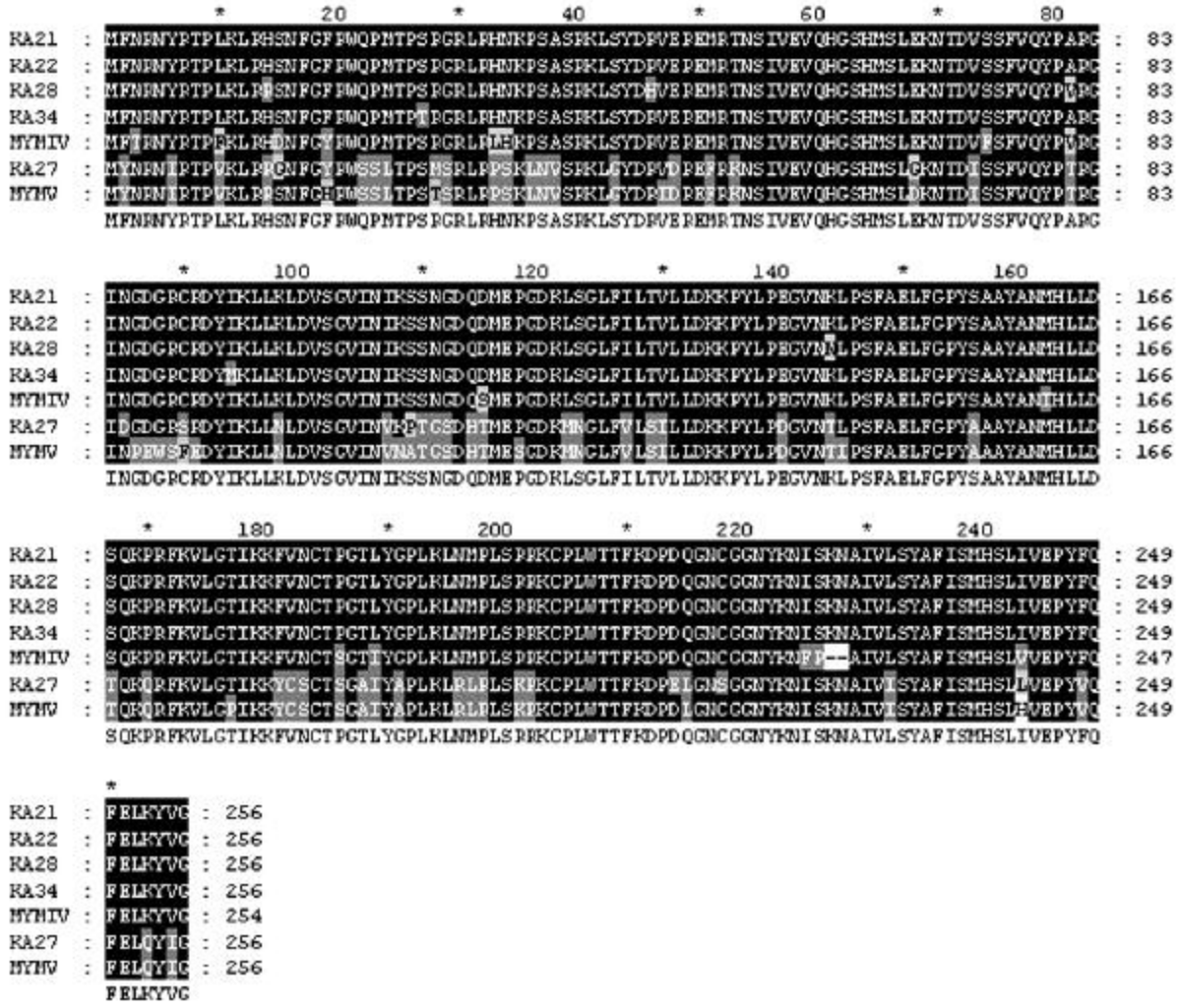

Figure 3. Alignment of the predicted amino acid sequences of the BV1 protein of MYMV-Vig DNA Bs (KA21, KA22, KA27, KA28 and KA34) with those of MYMIV and MYMV. Amino acids which are different from the consensus are highlighted as gray boxes. Dashes indicate gaps introduced for alignment.

A comparable trend is seen in DNA B titre as well. Upon agroinoculation with KA22 DNA B, the intensity in 0·04 μg sample of *V. mungo* is equal to 0·2 μg sample of *V. radiata* (~ 5-fold difference) (figure 8A). A contrasting pattern is observed upon agroinoculation with KA27 DNA B. The intensity in 0·04 μg sample of *V. radiata* is 2-fold higher than the intensity of 0·2 μg sample of *V. mungo* (~ 10-fold difference) (figure 8B).

From these results, it is evident that there is a strong correlation between the intensity of yellow mosaic symptoms induced by DNA Bs (KA22 and KA27) and the viral titre. KA22 DNA B caused typical yellow mosaic symptoms in *V. mungo* and the viral titre was high. In contrast, KA27 DNA B caused mild yellow mosaic symptoms in *V. mungo* and the viral titre was low. Interestingly, KA27 DNA B caused typical yellow mosaic symptoms in *V. radiata* and correspondingly virus titre was also high.

4. Discussion

The overall genomic organization of MYMV-Vig is equivalent to those of other geminiviruses with a bipartite genome (Stanley and Gay 1983; Stanley 1985; Lazarowitz 1992). The MYMV-Vig DNA A and five different DNA B components share a sequence identity of 85·6–95% (KA27–85·6% and KA22–95%) in the CR. The CR contains elements essential for replication and transcription (Hanley-Bowdoin *et al* 1999). A dsRNA cognate to the bidirec-

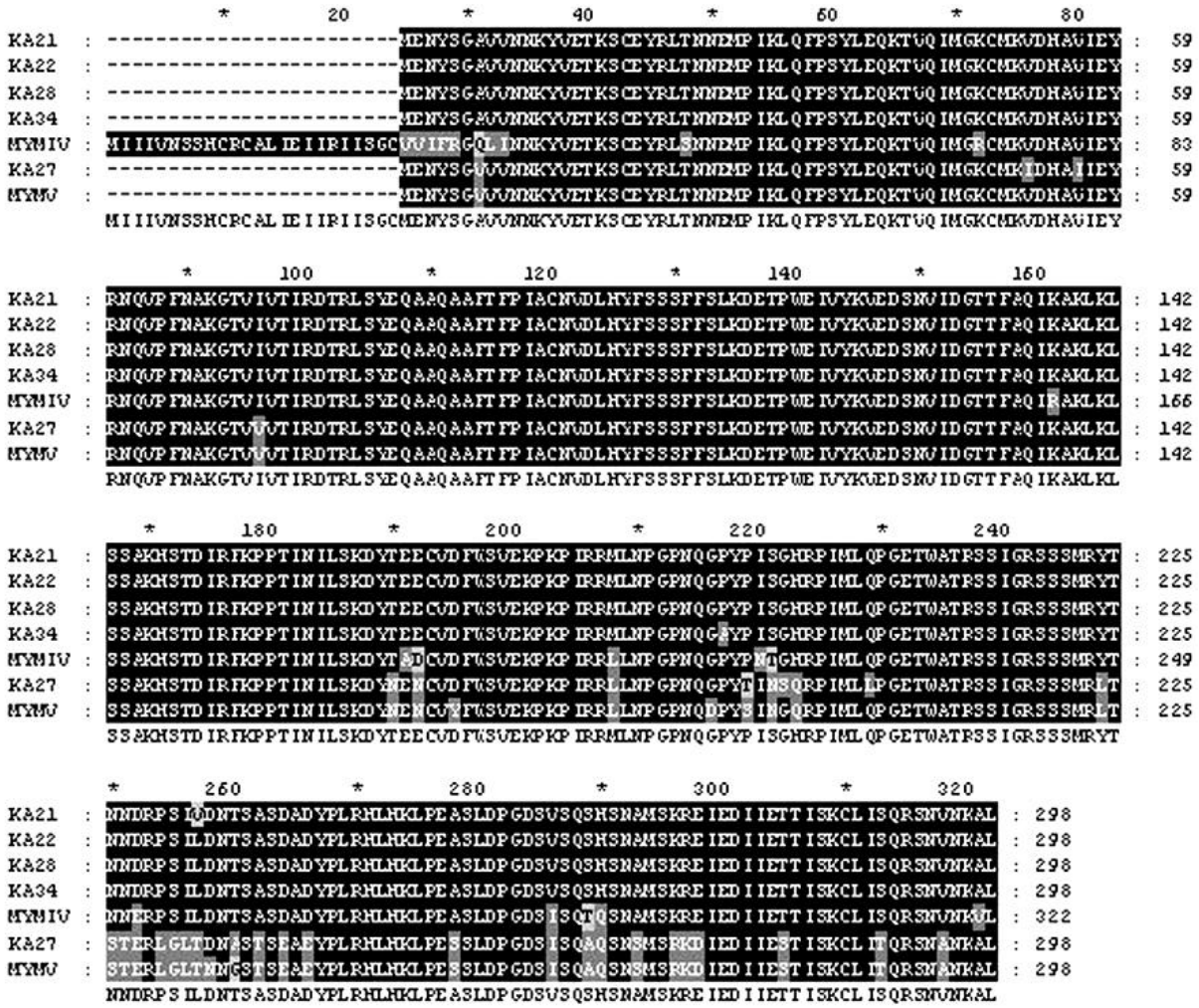

Figure 4. Alignment of the predicted amino acid sequences of the BC1 protein of DNA Bs of MYMV-Vig (KA21, KA22, KA27, KA28 and KA34) with those of MYMIV and MYMV. Amino acids which are different from the consensus are highlighted as gray boxes. Dashes indicate gaps introduced for alignment.

tional promoter (harbouring CR sequence) of MYMV-Vig DNA A was found to confer resistance in blackgram for MYMV-Vig (Pooggin *et al* 2003). Rep protein encoded in DNA A interacts with the CR of both DNA A and DNA B in a sequence-specific manner (Fontes *et al* 1992; Lazarowitz *et al* 1992; Behjatnia *et al* 1998). These specific binding sites are the iterons with lengths ranging from 8–12-nt (Arguello-Astorga *et al* 1994b). The 5′-TGTATCGGTGT-3′ (11-nt) sequence present as three direct repeats in the CR of MYMV-Vig is proposed to constitute the iterons. MYMIV had an iteron organization different from those of KA22 and KA27 DNA Bs of MYMV-Vig. However, the binding of MYMIV Rep protein to CR sequences in a specific manner has been demonstrated (Pant *et al* 2001). The CRs of DNA A and four DNA Bs (KA21, KA22, KA28 and KA34) revealed the existence of three 11-nt iterons. Interestingly, the KA27 DNA B showed a deletion of 3-nt in the first iteron (5′-ATCGGTGT) and carried an additional 18-nt sequence located between the third iteron and stem-loop structure. Surprisingly, both features (3-nt deletion in first iteron and the 18-nt insertion) were also found in the CR of MYMV DNA B (Morinaga *et al* 1993) reported from Thailand.

A comparison of amino acid sequences of BV1 and BC1 further strengthens the close relationship between MYMV-Vig KA27 DNA B and that of MYMV. In BC1, MYMV-Vig KA27 DNA B differs from other four DNA Bs in 29 amino acid positions. In BV1, it differs by 53 amino acids. Strikingly, the MYMV-Vig KA27 DNA B and MYMV DNA B are conserved in the positions of all the 82 amino acids mentioned above. Analysis of NLS, NES and phosphorylation sites showed that KA27 DNA B is closely related to MYMV.

A strong similarity of MYMV-Vig KA27 DNA B to MYMV DNA B cloned from infected *V. radiata* (mungbean) plants in Thailand (Morinaga *et al* 1993), prompted us to address the question whether KA27 DNA B is more adapted to infect *V. radiata*. KA22 DNA B caused typical yellow mosaic symptoms in *V. mungo* and mild yellow mosaic symptoms in *V. radiata* when agroinoculated along with the DNA A partial dimer. In contrast, KA27 DNA B induced mild yellow mosaic symptoms in *V. mungo* and typical yellow mosaic symptoms in *V. radiata*. The difference in the nature of symptoms induced by KA22 and KA27 DNA Bs (in combination with the same DNA A) in *V. mungo* and *V. radiata*, clearly indicates that the DNA

VirD2	P	K	R	P	R	D	R	M	D	G	E	L	G	G	R	K
KA21 BV1	P	S	R	G	R	L	R	M	N	K	P	S	A	S	R	K
KA22 BV1	P	S	R	G	R	L	R	M	N	K	P	S	A	S	R	K
KA28 BV1	P	S	R	G	R	L	R	M	N	K	P	S	A	S	R	K
KA34 BV1	P	T	R	G	R	L	R	M	N	K	P	S	A	S	R	K
KA27 BV1	P	S	M	S	R	L	R	P	S	K	L	N	V	S	R	K
MYMV BV1	P	S	T	S	R	L	R	P	S	K	L	N	V	S	R	K

Figure 5. Alignment of the nuclear localization signal (NLS) of MYMV-Vig BV1 proteins with that of *Agrobacterium* VirD2 protein. Bold types indicate the amino acids which are conserved.

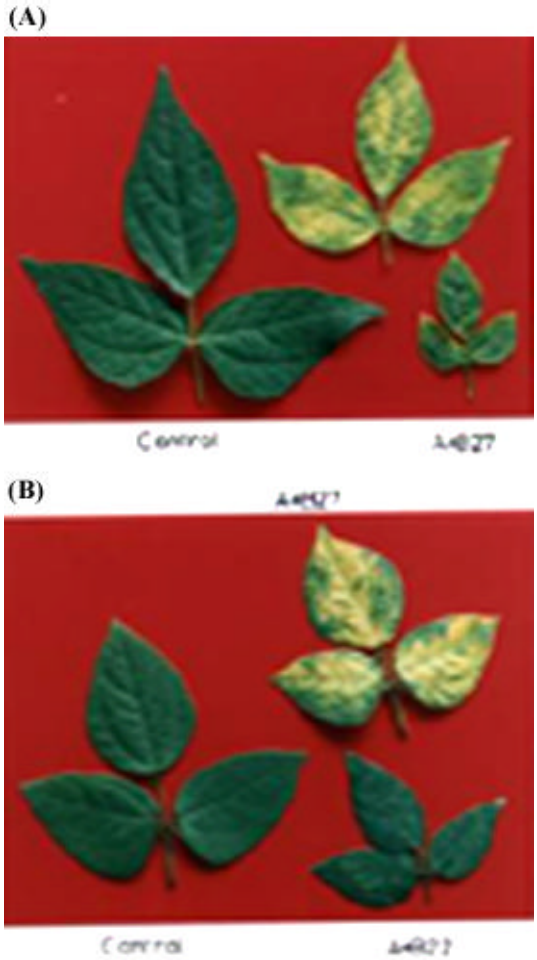

Figure 6. Agroinfection analysis of MYMV-Vig DNA Bs (KA22 and KA27) in *V. mungo* (**A**) and *V. radiata* (**B**). (**A**) Trifoliate leaf of *V. mungo* showing a typical yellow mosaic symptom upon agroinoculation with KA22 DNA B (pGA1·9B22) partial dimer clone and a mild yellow mosaic symptom upon agroinoculation with KA27 DNA B (pGA1·5B27) partial dimer in combination with the partial dimer clone of DNA A (pGA1·9A). A trifoliate leaf of mock-inoculated *V. mungo* (control) is shown. (**B**) Trifoliate leaf of *V. radiata* showing a typical yellow mosaic symptom upon agroinoculation with MYMV-Vig DNA A plus KA27 DNA B (pGA1·5B27) partial dimer clones and a mild yellow mosaic symptom upon agroinoculation with MYMV-Vig DNA A and KA22 DNA B partial dimers. A trifoliate leaf of mock-inoculated *V. radiata* (control) is shown. Photographs were taken 26 days after agroinoculation.

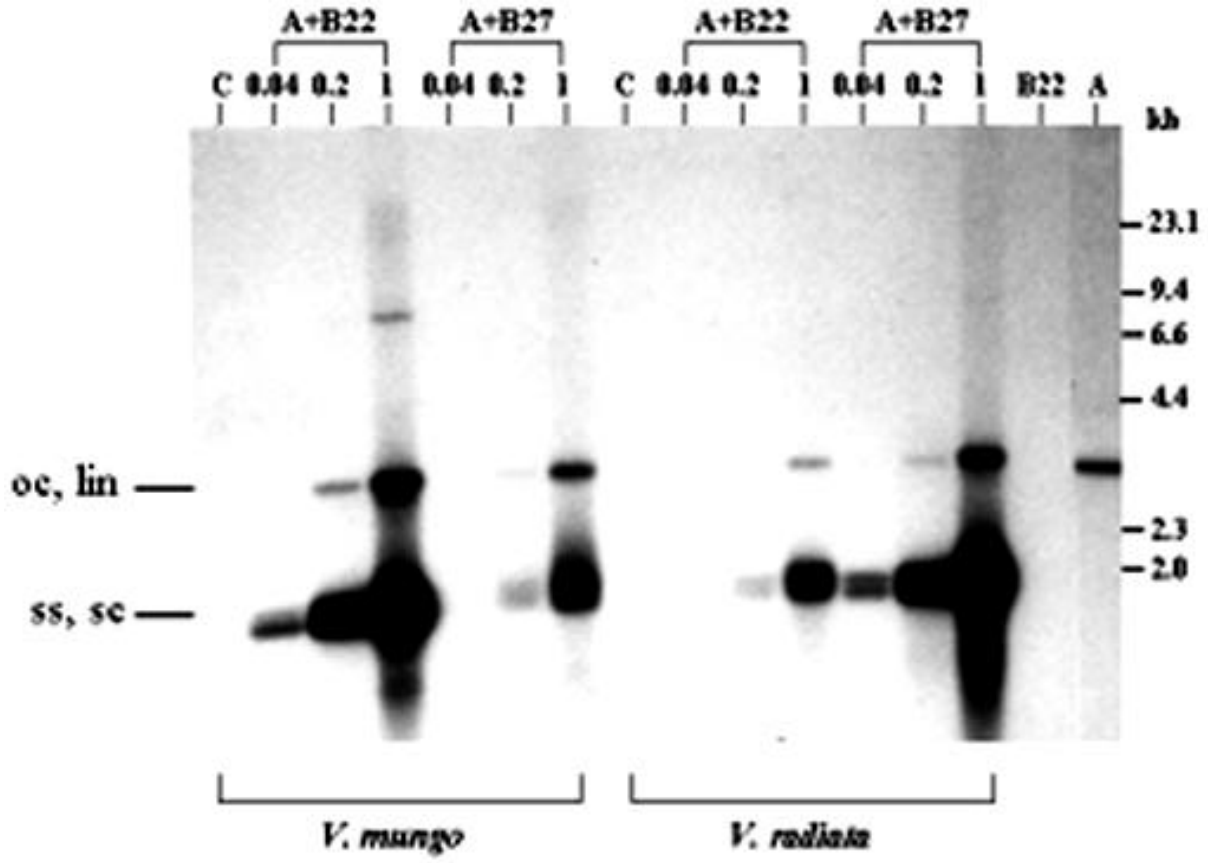

Figure 7. Southern blot analysis to compare relative levels of DNA A accumulation in *V. mungo* and *V. radiata* argoinoculated with a combination of DNA A and each of the two DNA Bs, KA22 (A + B22) and KA27 (A + B27). Total DNA samples from agroinoculated *V. mungo* and *V. radiata* plants were loaded at three sample sizes (0·04, 0·2 and 1 μg). Total DNA samples (1 μg) from mock-inoculated *V. mungo* and *V. radiata* were included as negative controls (C). A 1·2 kb *Bam*HI fragment of pKA30 (DNA A clone) devoid of CR was used as the probe. About 250 pg of full-length 2·7 kb DNA A (A) was loaded as the positive control. About 250 pg of full-length 2·7 kb KA22 DNA B (B22) was included in the blot as an internal negative control to ensure the absence of cross-hybridization between DNA A and DNA B.

B components are responsible for symptom determination. Difference in symptom development in two strains of TGMV (common strain and yellow vein strain) is attributed to the DNA B components of the two strains (Von Arnim and Stanley 1992). In contrast, among the two strains of ToLCNDV (severe strain and mild strain), the DNA A component was found to be responsible for the severe or mild leaf curl symptom (Padidam *et al* 1995; Chatterji *et al* 1999).

DNA A titre was higher in *V. mungo* when KA22 DNA B was used for agroinoculation. In contrast, DNA A titre was higher in *V. radiata* when KA27 DNA B was used for agroinoculation. KA22 DNA B titre was high in *V. mungo* and KA27 DNA B titre was high in *V. radiata*. Thus, a clear correlation was found between viral DNA accumulation and intensity of yellow mosaic symptom. Similarly, Chatterji *et al* (1999) observed a higher viral titre in plants infected with the severe strain of ToLCNDV compared to the mild strain.

Conclusively, KA22 DNA B is well adapted to infect *V. mungo* and the viral titre is also high. In contrast, KA27 DNA B is well adapted to infect *V. radiata* and the viral titre is also high. On the basis of sequence identity, CR organization, symptom development upon agroinoculation and viral titre, we propose that KA27 DNA B is more infective in *V. radiata* (mungbean) and is closely related to the mungbean isolate MYMV. Better adaptation of KA27 DNA B to *V. radiata* and high sequence identity (nucleotide-95%; BV1–94% and BC1–97·6%) it shares with MYMV DNA B (Morinaga *et al* 1993) raises interesting questions on the evolution of MYMV in South India and in Thailand.

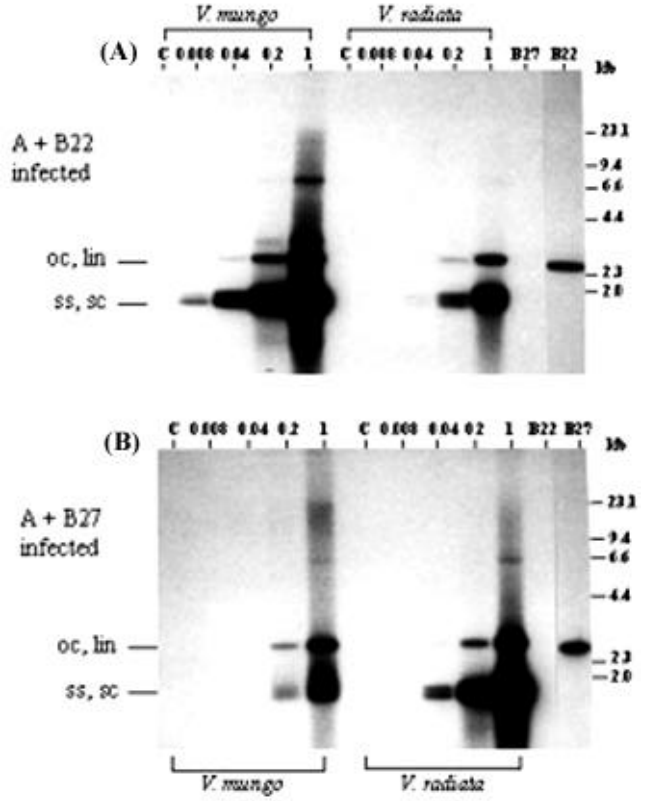

Figure 8. Southern blot analysis to compare relative levels of KA22 DNA B (**A**) and KA27 DNA B (**B**) in agroinoculated *V. mungo* and *V. radiata* plants. (**A**) Total DNA samples from *V. mungo* and *V. radiata* agroinoculated with partial dimers of MYMV-Vig DNA A and KA22 DNA B (A + B22) were loaded in four sample sizes (0·008, 0·04, 0·2 and 1 μg) with a 5-fold difference between them. (**B**) Total DNA samples from *V. mungo* and *V. radiata* agroinoculated with partial dimers of DNA A and KA27 DNA B (A + B27) were loaded in four sample sizes (0·008, 0·04, 0·2 and 1 μg). About 1 μg of total DNA samples from mock-inoculated *V. mungo* and *V. radiata* were included as negative controls (C) in both (**A**) and (**B**). A 1·0 kb *Bam*HI/*Cla*I fragment of pKA22 (DNA B clone) was used as probe in (**A**). A 1·3 kb *Hin*dIII fragment of pKA27 (DNA B clone) was used as probe in (**B**). Both probes were devoid of CR. In (**A**), about 250 pg of full-length 2·7 kb KA22 DNA B (B22) was used as the positive control. About 250 pg of full-length 2·7 kb KA27 DNA B (B27) was included in the blot as an internal negative control to ensure the absence of cross-hybridization between KA22 and KA27 DNA Bs. In (**B**), about 250 pg of full-length 2·7 kb KA27 DNA B (B27) and 2·7 kb KA22 DNA B (B22) were included as positive and negative controls, respectively.

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