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Analysis of an isolate of *Mungbean yellow mosaic virus* (MYMV) with a highly variable DNA B component

Brief Report

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Summary. One DNA A (KA30) and five different DNA B components (KA21, KA22, KA27, KA28 and KA34) of a geminivirus, Mungbean yellow mosaic virus–Vigna (MYMV-Vig) were cloned from a pooled sample of field-infected *Vigna mungo* plants from Vamban, South India. MYMV-Vig DNA A (KA30) and one of the DNA B components (KA27) exhibited 97% and 95% sequence identities, respectively, to those of MYMV reported from Thailand. However, the DNA B components KA21, KA22, KA28 and KA34 exhibited only 71 to 72% sequence identity to MYMV DNA B. Co-existence of multiple DNA B components in field-infected *V. mungo* was proved by Southern and PCR analyses. Each of the five DNA B components was infective together with the DNA A upon agroinoculation. Agroinoculation with mixed cultures of *Agrobacterium* with partial dimers of DNA A and all five DNA Bs proved that all five DNA B components can co-infect a single *V. mungo* plant.

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Mungbean yellow mosaic virus (MYMV) infects five important pulse crops, blackgram, mungbean, Frenchbean, pigeonpea and soybean, causing an annual yield

Note: The nucleotide sequences reported in this paper have been deposited in the EMBL/GenBank/DDJB Nucleotide Sequence Database under the accession numbers AJ132575 (DNA A), AJ439059 (KA21 DNA B), AJ132574 (KA22 DNA B), AF262064 (KA27 DNA B), AJ439058 (KA28 DNA B) and AJ439057 (KA34 DNA B).

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loss of about $300 million [16]. Yellow mosaic disease in blackgram and mungbean caused by MYMV was first reported in 1960 [6]. MYMV belongs to the genus *Begomovirus* under the family *Geminiviridae* [2]. Molecular cloning of MYMV isolates from Thailand (MYMV) [5] and Vamban, South India (MYMV-Vig) [15] as well as a closely related species from New Delhi, India (MYMIV) [4] has shown that the genomes of these viruses are bipartite. A recent reassessment of the criteria for distinguishing species within the genus *Begomovirus* has reclassified the virus previously known as Vigna mungo yellow mosaic virus [8, 15] as a strain of MYMV (MYMV-Vig) [2].

Total DNA was extracted [10] from a pooled sample of young leaves of many symptomatic *V. mungo* plants collected at one time from the experimental field of National Pulses Research Centre, Vamban, Tamil Nadu, India. The DNA was electrophoresed in 1× TNE buffer (40 mM Tris-acetate, pH 7.5, 20 mM sodium acetate and 2 mM EDTA). Following ethidium bromide staining, a ∼2.7-kb band corresponding to the replicative form (RF) was gel-purified. Sub samples of DNA were digested with *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I and *Xba*I and cloned into pOK12 [17]. Fourteen putative full-length clones obtained were grouped into six types on the basis of restriction endonuclease digestion patterns. Among these, one (KA30) was identified as a DNA A clone on the basis of its hybridization to *Indian cassava mosaic virus* (ICMV) DNA A. The remaining five clones (pKA21, pKA22, pKA27, pKA28 and pKA34) were assumed to be DNA Bs since they did not hybridize with ICMV DNA A but did hybridize to the purified RF and to the DNA A of MYMV-Vig (KA30) by virtue of their shared common region (CR) sequence. Restriction analysis of the five DNA B clones revealed distinct restriction patterns (data not shown).

The nucleotide sequences of pKA30, pKA22 and pKA27 were determined manually by the dideoxy chain termination method [12]. Clones pKA21, pKA28 and pKA34 were sequenced in a ABI Prism 310 Gene Analyzer (Applied Biosystems, USA). All the clones were sequenced in both directions. The length of the DNA A component is 2725 nt (accession number AJ132575). The length of DNA Bs are, KA21-2656 nt (AJ439059), KA22-2660 nt (AJ132574), KA27-2676 nt (AF262064), KA28-2656 nt (AJ439058) and KA34-2660 nt (AJ439057).

To explore whether one or multiple DNA A components are present in field-infected *V. mungo*, Southern analysis was performed by digesting total plant DNA with a range of restriction enzymes. DNA was alkali-denatured and transferred [13] to Zeta-probe nylon membrane (Bio-Rad Laboratories, USA). A DNA A-specific probe from pKA30 without CR was used. Probe DNAs were prepared using the Megaprime^{TM} DNA labeling system (Amersham International Plc. Ltd., UK) and [α-^{32}P]dCTP (BRIT, Mumbai, India). Total DNA digested with *Bgl*II, *Hin*cII, *Hin*dIII, *Nco*I and *Pst*I showed a 2.7-kb band representing the linearized DNA A RF (Fig. 1A). Two bands (0.9-kb and 1.8-kb) obtained with *Dra*I digestion correlated with the two sites in the DNA A RF. On the basis of Southern analysis, it was inferred that only one DNA A component existed in the field-infected *V. mungo* plants.

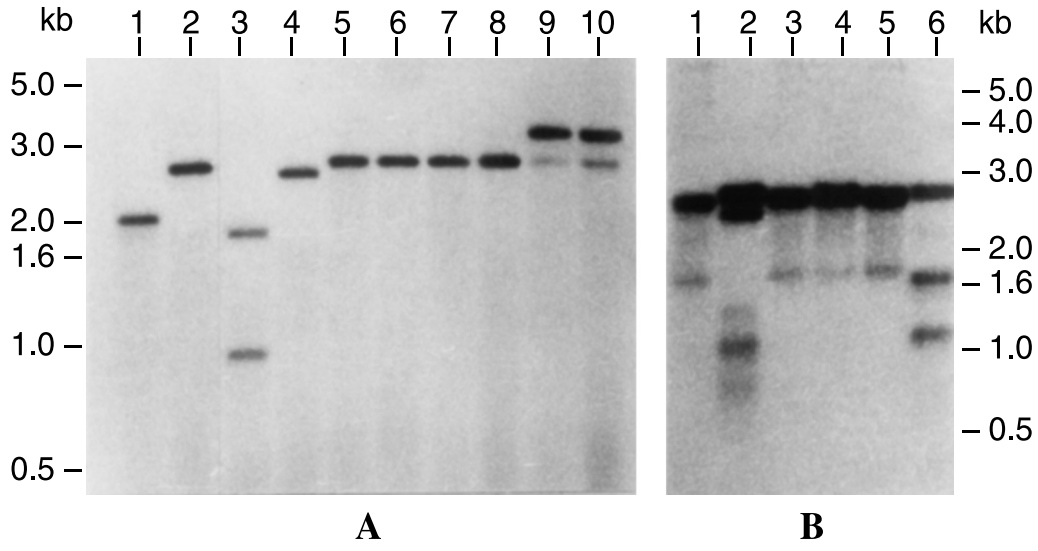

Fig. 1. Southern hybridization analysis of total DNA extracted from infected, field-collected *V. mungo* plants. **A** DNA A without CR was used as the probe. **B** KA34 DNA B (devoid of CR) was used as the probe. In Section **A**, total DNA (1 μg) was digested with *Bam*HI (*1*), *Bgl*II (*2*), *Dra*I (*3*), *Eco*RI (*4*), *Hin*cII (*5*), *Hin*dIII (*6*), *Nco*I (*7*), *Pst*I (*8*) and *Xba*I (*9*). The digested samples along with undigested total DNA (lane 10) were separated in a 1.2% agarose gel (TBE buffer), transferred to a nylon membrane and hybridized to a DNA A-specific probe (0.74-kb plus 1.3-kb *Bam*HI/*Pst*I fragments of pKA30). The 0.18-kb *Eco*RI fragment was detected upon longer exposure (not shown here). In Section **B**, total DNA (1 μg) restricted with *Bam*HI (*2*), *Eco*RI (*3*), *Hin*dIII (*4*), *Pst*I (*5*) and *Xba*I (*6*) were separated in a 1% agarose gel (1X TNE buffer) along with uncut DNA (*1*), transferred to a nylon membrane and hybridized to a DNA B-specific probe (2.1-kb *Cla*I fragment of pKA34 DNA B clone). The 0.3-kb *Bam*HI fragment was detected in *2* upon longer exposure (not shown here)

To confirm the presence of multiple DNA B components in field-infected *V. mungo*, Southern analysis was performed by digesting the total DNA with restriction enzymes which could differentiate the five DNA B components. A DNA B-specific probe (2.1-kb *Cla*I fragment of pKA34) without CR was used. Total DNA digested with *Bam*HI showed three bands of expected sizes (2.7-kb, 2.4-kb and 0.3-kb) (Fig. 1B). Presence of undigested DNA between 2.7-kb and 2.4-kb bands could not be completely ruled out. The 2.7-kb fragment represents the linearized RF of KA21 or KA28 DNA Bs. The 2.4-kb and 0.3-kb fragments represent those of KA22 or KA34. One intense band at 1.0-kb and two light bands at 1.3-kb and 0.7-kb positions could not be explained based upon the five cloned DNA B components. With *Xba*I digestion, the 2.7-kb fragment indicated the presence of KA21, whereas the 1.1-kb and 1.5-kb bands together may be derived from KA22, KA28 or KA34 (Fig. 1B). A 1.4-kb *Hin*dIII band that corresponds to KA34 is, however, not seen in this analysis. Under the high stringency washing conditions (0.1× SSC, 65 °C), the KA34 probe did not hybridize to KA27 DNA B. The presence of KA27 DNA B in field-infected *V. mungo* was detected in a separate Southern in which a 1.4-kb *Hin*dIII fragment of KA27 (without the

CR) was used as a probe under high stringency washing conditions (result not shown).

The sizes of fragments obtained with *Bam*HI and *Xba*I restriction correlated well with the restriction patterns of the three DNA Bs (KA21, KA22 and KA28). The combined length of all fragments produced by digestion with one enzyme was more than 2.7-kb, suggesting the existence of more than one DNA B component. An independent field-infected *V. mungo* sample was collected from the same experimental field after five years and was analyzed by Southern to check whether multiple DNA Bs co-existed consistently. The same restriction pattern for DNA Bs was seen in the sample collected after a five-year period.

A PCR-based approach was additionally taken to confirm the co-existence of multiple DNA B components of MYMV-Vig in field-infected samples. Two primers were designed to conserved sequences within the coding regions of the movement protein (nucleotide numbers 2101 to 2124 of KA22) and nuclear shuttle protein (608 to 590 of KA22). A fragment of 1.1-kb is expected to be amplified from all DNA Bs. The amplified 1.1-kb fragment was gel-purified and cloned in pGEM-T (Promega, Madison, U.S.A.). Eight clones were picked randomly and sequenced. The sequences of four clones matched with that of KA21 DNA B, two matched with KA22 DNA B sequence and two matched with KA28 DNA B sequence (data not shown).

Genetic distances were used to construct phylogenetic trees (Fig. 2). Phylogenetic analysis was done by the neighbour-joining method [11] and its reliability was assessed by bootstrapping [3] with 1000 replications. Phylogenetic trees were drawn using 'njplot' tree drawing program [7]. The DNA A phylogram (Fig. 2A) shows that MYMV-Vig formed a tight cluster with MYMV-Sb [Mad] and they branched along with MYMV-Vig [Mah] and MYMV. DNA B phylogram (Fig. 2B) showed that KA22 DNA B (along with KA21, KA28 and KA34) branched along with MYMIV-[Sb] and MYMIV-[Cp]. However, KA27 DNA B of MYMV-Vig, which is distinct from the other four DNA Bs, clustered tightly with MYMV (Fig. 2B).

Nucleotide sequence identities of MYMV DNA A to those of MYMV-Vig, MYMV-Vig [Mah] and MYMV-Sb [Mad] are 97%, 98.2% and 98.4%, respectively. On the basis of a sequence identity of more than 89% [2], MYMV, MYMV-Vig, MYMV-Vig [Mah] and MYMV-Sb [Mad] are considered as strains of one species. MYMIV constitutes a separate species since it has a sequence identity of less than 89% (the cut off for distinguishing species from strains for begomoviruses [2]) to MYMV (81.3%).

The KA27 DNA B of MYMV-Vig has 95% sequence identity to MYMV DNA B. However, the MYMV DNA Bs KA21, KA22, KA28 and KA34 exhibit sequence identity to MYMV DNA B in the range of 71 to 72%. MYMIV DNA B has only 70% sequence identity to MYMV DNA B. Interestingly, the MYMV-Vig DNA Bs (KA21, KA22, KA28 and KA34) exhibit 89 to 90% sequence identity to MYMIV. The sequence identities of the movement protein and the nuclear shuttle protein of MYMV-Vig and MYMIV are 96% and 95%, respectively. The sequence identities reported here suggest a possible psuedo-recombination event (involving

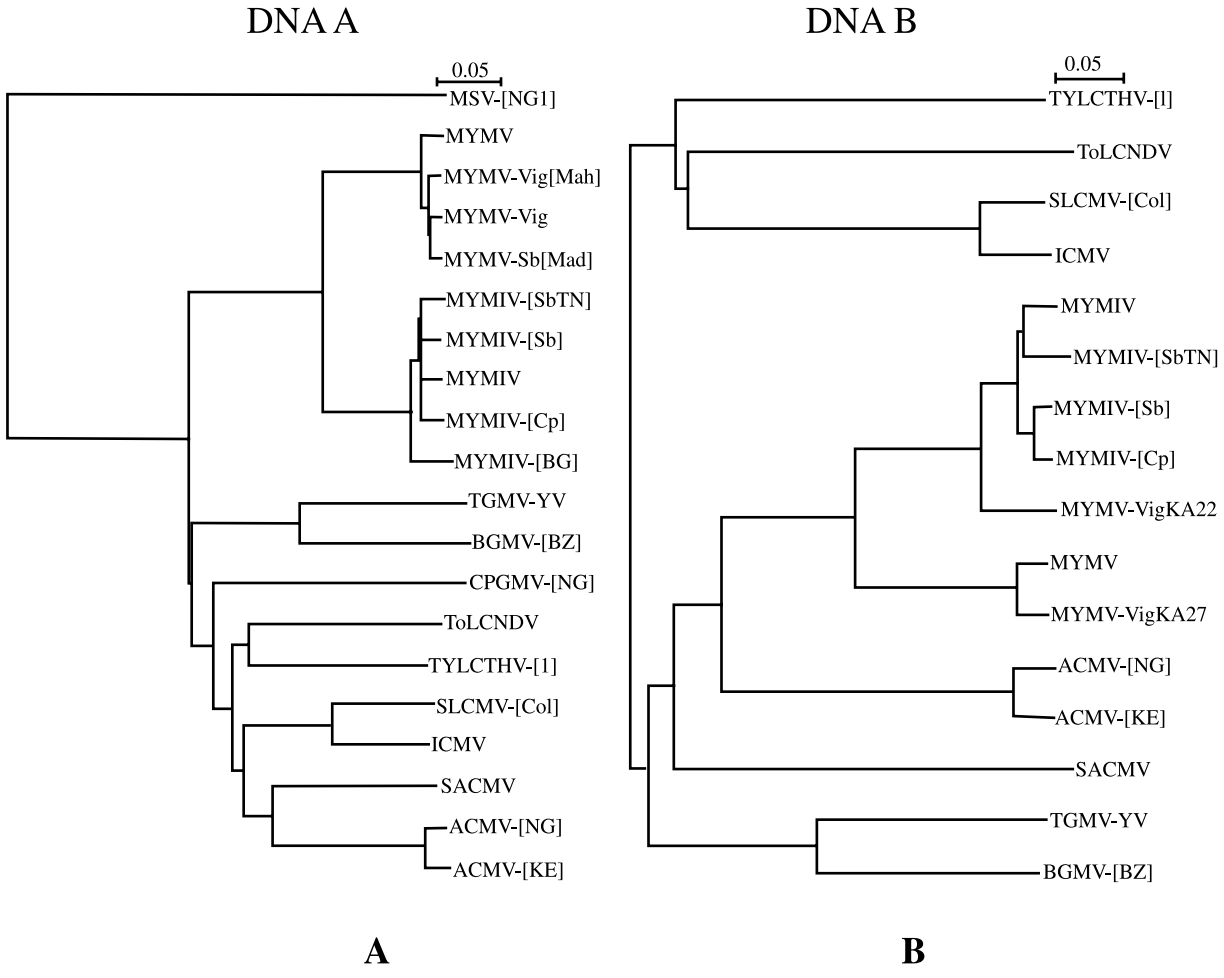

Fig. 2. Phylogram of DNA A (**A**) and DNA B (**B**) sequences (KA22 and KA27) of MYMV-Vig and other begomoviruses. *Maize streak virus* (Nigerian isolate) was used as an outgroup for the DNA A phylogram. The phylograms were constructed using the neighbour-joining and bootstrap (1000 replications) options of CLUSTAL W. Vertical distances are arbitrary and horizontal distances reflect number of nucleotide differences between branch nodes. Accession numbers and nomenclatures of viruses are as per the latest revision of virus nomenclature [2]

MYMV DNA A and MYMIV DNA B) contributing to the emergence of the variant form MYMV-Vig. The five DNA Bs of MYMV-Vig exhibit considerable differences in their sequences and constitute two distinct groups; one comprising KA27 and the second comprising KA21, KA22, KA28 and KA34.

Agroinoculation of cloned genomic components of MYMV-Vig was done to check whether each of the five DNA B components can cause the yellow mosaic disease along with one DNA A component. Partial dimeric clones of the DNA A (KA30) and five DNA Bs (KA21, KA22, KA27, KA28 and KA34) of MYMV-Vig, each containing two origins of replication as direct repeats, were

constructed in a modified version of the binary vector pGA472 [1] using the restriction sites identified in each clone. The partial dimer clones were introduced into *Agrobacterium tumefaciens* strain C58 either by triparental mating or by electroporation. *V. mungo* cv Co5 seedlings were agroinoculated as described by Mandal et al. [4].

Mock inoculated *V. mungo* plants and those agroinoculated only with DNA A or DNA B partial dimers did not develop symptoms of the disease. Typical yellow mosaic symptoms were observed (Fig. 3) in the trifoliate leaves two weeks after agroinoculation with *A. tumefaciens* strain C58 harbouring the partial dimers of DNA A and each of the four DNA Bs (KA21, KA22, KA28 and KA34). However, KA27 DNA B, when agroinoculated with DNA A, induced mild yellow mosaic symptoms. In addition, the plants were stunted, the trifoliate leaves showed downward curling and the leaflets were smaller with shortened petioles. Each agroinoculation experiment was repeated and the nature of symptoms were comparable (Table 1). KA22 DNA B caused the highest percentage (91%) of infection.

Southern hybridization analysis of plants infected by agroinoculation was done to confirm that the yellow mosaic symptom was associated with accumulation of

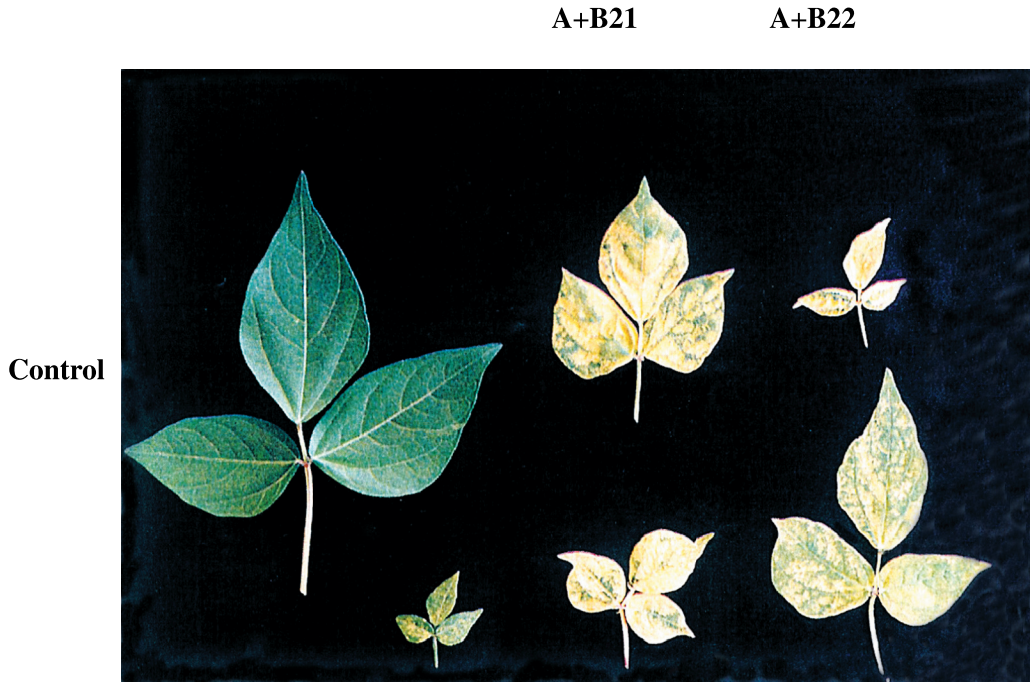

Fig. 3. Infectivity of MYMV-Vig clones by *Agrobacterium*-mediated inoculation. Trifoliate leaves of *V. mungo* plants showing yellow mosaic symptoms upon agroinoculation with partial dimers of DNA A and each of the five DNA Bs of MYMV-Vig. Symptoms appeared 14 days after agroinoculation. DNA A component is represented as A. B21, B22, B27, B28 and B34 denote the respective DNA B of MYMV-Vig

Table 1. Infectivity and symptom phenotype in *Vigna mungo* upon agroinoculation with cloned MYMV-Vig genomic components

DNA B	Plants infected/inoculated (%)		Symptom type
	Expt I	Expt II	
KA21	54/60 (90)	16/36 (44)	typical yellow mosaic
KA22	22/24 (92)	45/50 (90)	typical yellow mosaic
KA28	16/21 (76)	3/36 (8)	typical yellow mosaic
KA34	24/40 (65)	22/35 (63)	typical yellow mosaic
KA27	42/60 (70)	40/50 (80)	mild yellow mosaic

All agroinoculations involved DNA A and one of the DNA Bs

Agrobacterium tumefaciens strain C58 harbouring partial dimers of MYMV-Vig were used for agroinoculation

Infectivity was scored three weeks post-inoculation

viral DNA. Total DNA was extracted from upper leaves pooled from *V. mungo* plants inoculated with DNA A and one DNA B [9]. The ss- and ds-viral DNA were distinguished on the basis of S_1 nuclease sensitivity [14]. Both ss- and ds-viral DNA were present in plants agroinoculated with DNA A and DNA B combinations (results not shown). Thus, all DNA B components are infectious and a single DNA A functionally interacts with five different DNA B components of MYMV-Vig and efficiently *trans*-replicates them.

Based on the Southern analysis of pooled field samples, it was proposed that the five DNA B components of MYMV-Vig were present in a single plant. Since pooled samples were analyzed in those experiments, it was not possible to conclude that one infected plant carried multiple DNA Bs. To address this question, an experiment was done to simultaneously infect *V. mungo* plants with DNA A and five different DNA Bs of MYMV-Vig. *A. tumefaciens* cultures with partial dimer clones of DNA A (KA30) + DNA B-KA27, DNA B-KA21, DNA B-KA22, DNA B-KA28 and DNA B-KA34 were mixed in equal proportions and used for agroinoculation. Six out of 10 agroinoculated plants developed typical yellow mosaic symptoms. DNA was extracted [9] from the upper leaves of four plants showing symptoms. PCR was done with the total DNA extracted from all the four plants using KA27 (DNA B)-specific primers (forward – 5′-CCTGAATAATTCTCCATTATTCAG-3′, reverse – 5′-CCGGTTAGTAGGTCT AAAGTG-3′). All the four plants showed a 0.6-kb amplicon (Fig. 4A) corresponding to the KA27 DNA B sequence, thus confirming the presence of this DNA B in the infected plants. To confirm the presence of other DNA Bs, a Southern hybridization analysis was performed. Single stranded DNA was removed by digestion with S_1 nuclease [14]. The ds-DNA was digested with *Cla*I, *Hin*dIII and *Xba*I and separated in 1% agarose gels in 1X TBE buffer. The DNA was transferred to Zeta-probe membrane and hybridized to a 1.0-kb *Bam*HI/*Cla*I fragment of pKA22 (DNA B) without CR. The analysis of a representative plant infected with

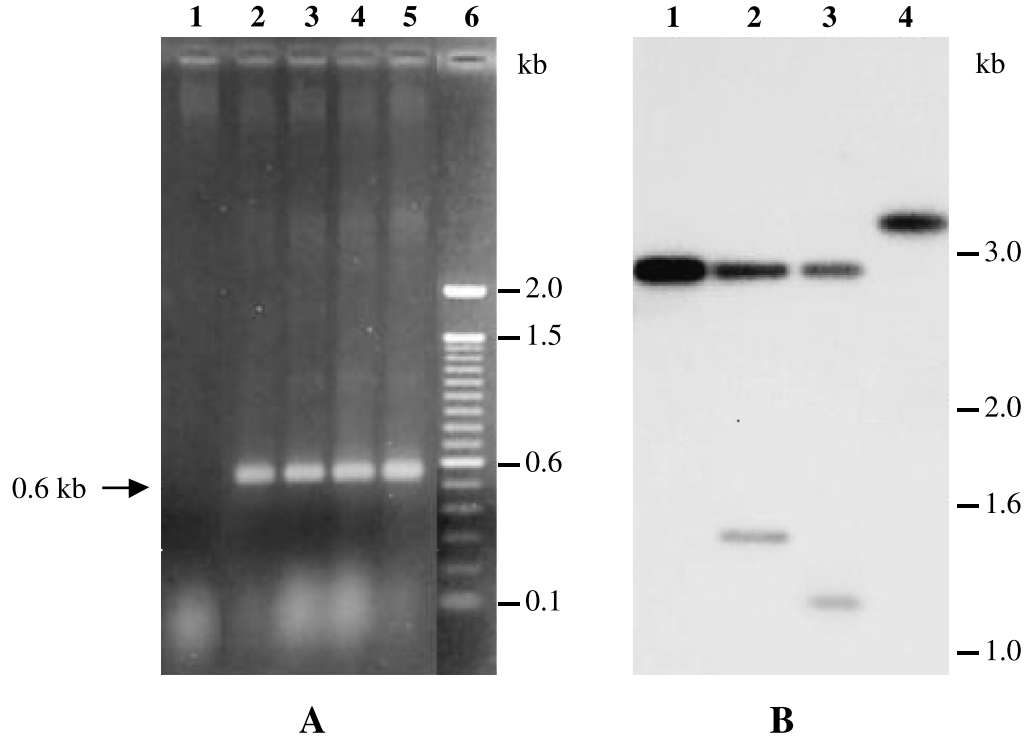

Fig. 4. Analysis of total DNA extracted from *V. mungo* plants coagroinoculated with a combination of partial dimer clones of DNA A and all five DNA Bs (KA21, KA22, KA27, KA28 and KA34). **A** PCR analysis of four coagroinoculated plants with KA27 DNA B-specific primers. Total plant DNA (100 ng) was used as template and PCR products were separated in a 1% agarose gel, stained with ethidium bromide. *1* had uninoculated plant DNA as control. *2*, *3*, *4* and *5* had templates from four individual plants (plant number 1, 2, 3 and 4, respectively) coagroinoculated with five different DNA Bs. A marker was loaded in lane *6* (100-bp ladder; Invitrogen, California, U.S.A.). The expected amplicon size is 0.6 kb (marked with an arrow). **B** S_1 nuclease-treated total DNA samples (500 ng) from plant number 4 were digested with *Cla*I (*1*), *Hin*dIII (*2*) and *Xba*I (*3*) and separated in a 1% agarose gel (1X TBE buffer), transferred to nylon membrane and hybridized to DNA B-specific (1.0-kb *Bam*HI/*Cla*I fragment of pKA22) probe devoid of CR. The probe was labeled with [α-^{32}P]dCTP. In lane 4, S_1 treated DNA was not digested with any restriction enzyme

all five DNA Bs is shown in Fig. 4B. The 2.7-kb *Cla*I fragment (lane 1) confirms KA28. The 2.7-kb and 1.4-kb *Hin*dIII fragments (lane 2) confirm KA22 and KA34, respectively. The 2.7-kb *Xba*I fragment (lane 3) confirms KA21. Therefore, the coagroinoculated plant carried all the five DNA Bs, supporting the idea that, in the field, one *V. mungo* plant can harbour one DNA A and five different DNA B components of MYMV-Vig.

The yellow mosaic disease in *V. mungo* is an interesting example of a mixed infection involving five DNA B components. Each of the five DNA B components of MYMV-Vig are replicated efficiently by one DNA A. The co-existence of five different DNA Bs along with DNA A is stable over a period exceeding five years.

Interestingly, coagroinoculation of individual *V. mungo* plants with DNA A and five DNA B clones resulted in the co-infection of all the five DNA B components.

Co-existence of multiple DNA B components of MYMV-Vig can be helpful in the extension of host range. The additional DNA B components may be variants that efficiently infect the related *Vigna* species such as *V. radiata* (mungbean) and *V. aconitifolia* (mothbean), but are replicated and maintained at low levels in *V. mungo*. Alternatively, the different DNA B components may complement each other and facilitate more effective movement within *V. mungo*. It is also likely that one DNA B may compete with the second DNA B and reduce the intensity of the disease. The implications of co-infection of multiple DNA B components in the yellow mosaic disease of *V. mungo* is an interesting question that needs further exploration.

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