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Diagnosis of Symptomless *Yellow mosaic begomovirus* Infection in Pigeonpea by Using Cloned *Mungbean yellow mosaic India virus* as Probe

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One isolate of *Mungbean yellow mosaic India virus* (MYMIV) of mungbean plants from Sri Ganganagar, Rajasthan, designated as MYMIV-Mg was isolated and DNA-A and DNA-B, the two full length bipartite genomic components of this virus, were cloned. The [α-^{32}P] labeled diagnostic probes specific to these cloned DNA-A and -B of MYMIV-Mg were used to detect the virus infection in infected plants by nucleic acid spot hybridization (NASH) test. The NASH tests detected the MYMIV infection and concentration of viral titre in susceptible, moderately susceptible, resistant and symptomless genotypes of pigeonpea (*Cajanus cajan*) plants. Fourteen genotypes of pigeonpea were tested against five naturally occurring MYMIV variants viz., MYMIV-Bg, -MgD, -MoL, -Mg and -Pp1 through viruliferous whitefly (*Bemisia tabaci*) transmission in greenhouse condition. Disease incidence and severity of MYMIV in different pigeonpea genotypes varied with the variants of MYMIV. Many genotypes of pigeonpea did not produce visible yellow mosaic symptoms after inoculation with MYMIV variants MYMIV-Bg, -MbD and -MoL, although, majority of the symptomless genotypes were found to be infected by MYMIV, as viral DNA was detected by NASH test.

Key words: *Cajanus cajan,* symptomless infection, *Mungbean yellow mosaic India virus*, nucleic acid spot hybridization.

Grain legumes are the economically important food crops in India. All the legumes are susceptible to numerous viruses of which yellow mosaic disease (YMD) caused by *Mungbean yellow mosaic India virus* (MYMIV) and *Mungbean yellow mosaic virus* (MYMV) is a serious constraint in production of pulses in India (1,2). MYMIV has been shown to have geminate particles measuring about 18 x 30 nm (3) and has a single stranded, circular DNA genome which consists of two split DNA species, designated as DNA-A and DNA-B of about 2.7 kb each (4,5). This virus is readily transmitted by whitefly (*Bemisia tabaci*) in semi-persistent manner but not by sap and seed (6).

Pigeonpea (*Cajanus cajan*) is an important drought resistant food crop, grown on area of about 3.8 million hectares in Indian subcontinent with annual production of 3 million tonnes. Yellow mosaic in pigeonpea was first reported in Delhi, Uttar Pradesh and Andhra Pradesh (7) and the crop is considered as one of the important hosts of MYMV in cultivated legumes (8). Recently, an increasing rate of YMD incidence in pigeonpea at several agro-climatic zones of India has been observed. On the basis of field and greenhouse screening for the symptoms and disease severity, a large number of genotypes of several pulse crops have been reported to be resistant to YMD (2, 9-11). But, screening for resistance to ymd in pigeonpea has not been performed in India, therefore, no resistant genotypes in pigeonpea to this disease have been reported so far.

The virus isolates causing yellow mosaic diseases in pulses have been characterized in recent years, and presently in India two species of yellow mosaic viruses, one (MYMIV) prevailing in Northern and Central parts of India and other (MYMV) in Southern and Western parts of India are recognized, and both the species belong to the genus *Begomovirus* of the family *Geminiviridae* (12,13). Detection of viruses in legumes by molecular methods is of paramount importance to formulate strategy for the management of viral diseases. Further, symptomless infection caused by different viruses has been reported in many crops like mungbean, blackgram and chilli (10, 11, 14,15). The symptomless hosts serve as reservoir of acquisition and transmission of viruses to susceptible crop plants, which in turn induce symptoms under suitable

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Abbreviations: MYMIV- *Mungbean yellow mosaic India virus*; AAP-acquisition access period; IAP-inoculation access period; NASH-nucleic acid spot hybridization.

environmental conditions causing crop loss. Therefore, detection of viral infection in symptomless host is essential, and this should be possible by advanced molecular methods.

In the present investigation, disease reaction of different genotypes of pigeonpea was studied against five variants of MYMIV in greenhouse, and one MYMIV variant from mungbean plant was cloned and its full length genome was used as diagnostic DNA probe for nucleic acid spot hybridization (NASH) test to identify MYMIV infection in diseased and symptomless pigeonpea plants.

Materials and Methods

Sources of cultures — The five MYMIV variants used in present study were obtained from different parts of India. For instance, MYMIV-Pp1 from infected pigeonpea plants, Delhi, -Bg from blackgram, Delhi, -MgD from mungbean (*Vigna radiata*), Delhi, -MoL from mothbean (*V. aconitifolia*), Ludhiana and –Mg from mungbean, Sriganganagar, Rajasthan originally characterized biologically by Biswas and Varma (14), were maintained in susceptible blackgram (*Vigna mungo*) *cv* Pusa 2 through whitefly transmission in insect-proof greenhouse.

Whiteflies used in this study were adult populations and maintained on healthy tobacco (*Nicotiana tabacum cv* Xanthi and White Burley) plants during the winter months and on healthy bottle gourd (*Lagenaria siceraria*) *cv* Pusa Naveen and blackgram *cv* Pusa 2 during the summer months by serial transfer. These plants were kept covered with muslin cloth in insect-proof greenhouse.

Seeds of the fourteen genotypes of pigeonpea and other genotypes of blackgram and mungbean were obtained from the Division of Genetics of our Institute.

Whitefly inoculation of MYMIV to plant — Adult whiteflies were given acquisition access period (AAP) for 24 h on infected plants of blackgram *cv* Pusa 2 and then released on healthy test plants at two-leaf stage for inoculation access period (IAP) of 24 h. Pigeonpea plants were gown in 25 cm diameter earthen pots covered with muslin cloth in insect-proof greenhouse. For five MYMIV variants, five separate greenhouse chambers were used. Test plants were kept for IAP by releasing 8-10 viruliferous whiteflies/ plant at two-leaf stage (3 days after germination) using small plastic cages which could cover each plant individually. After IAP, whiteflies were killed by spraying insecticide with recommended dose, and the plants were kept in an insect-proof greenhouse for six weeks for observation of symptoms and collection of samples for studying infection by NASH test.

Observations on disease severity — The severity of YMD in pigeonpea and mungbean was recorded according to the visual symptoms after six weeks post-inoculation using a 0-3 scale, where; 0, symptomless or immune; 1, resistant (mild symptoms, 0.1- 5% of leaf area showing symptoms, producing small yellow spots scattered); 2, moderately resistant (5.1- 25% of leaf area showing symptoms, yellow spots to small patches); 3, susceptible (25.1- 50% of leaf area showing symptoms, yellow spots, small to large yellow patches).

Molecular cloning — For isolation of total nucleic acids, MYMIV-Mg infected symptomatic leaves of fourteen days old young plants of blackgram *cv* Pusa 2 were harvested, ground in liquid nitrogen, and homogenized in extraction buffer (100 mM Tris-Cl, pH 8.0; 100 mM NaCl; 10 mM EDTA; 0.05% SDS). The slurry was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was transferred into centrifuge tubes and emulsified with equal volume of phenol: chloroform: isoamylalcohol (25:24:1). The mixture was centrifuged at 15000 rpm for 20 min. The aqueous phase was collected and 2.5 volume of absolute alcohol and 1/10th volume of 3M sodium acetate (NaOAc), pH 4.8 was added to it, mixed gently and left overnight at 20°C. The precipitate was pelletized by centrifuging at 20,000 rpm for 20 min, washed with 70% alcohol, dried and dissolved in resuspension buffer (10 mM Tris-Cl, pH 7.4; 1 mM EDTA). Double standard viral replicative forms were separated from the host DNA by cesium chloride (CsCl) density gradient centrifugation (4). After centrifugation, the centrifuge tubes were pierced near the bottom end and 20 aliquots of about 200 µl each were collected in eppendorf tubes from bottom end upto the host chromosomal band. Each fraction was individually extracted with n butanol saturated with 4M NaCl for three to four times until entire EtBr was removed. CsCl was removed by dialysis against sterilized water. The DNA was precipitated by adding 2.5 volume of ethanol and 1/10th volume of 3M NaOAc, pH 4.8, mixed and kept at 20°C for 1 h. The precipitate was collected after centrifugation at 15,000 rpm for 30 min and washed with 70% alcohol. The DNA pellet was dried and dissolved in 20 µl sterile double distilled water (ddw) and kept at 20°C. DNA (2 µl) from each of the 20 fractions was tested in 1% agarose gel electrophoresis using 1x TAE

buffer containing EtBr (0.5 μg ml^{-1}). The viral DNA was cloned at *Pst* I site of pUC18 plasmid vector (Bangalore Genei India Ltd.), and then *E. coli* strain NM522 was transmitted with the plasmid following the methods as described earlier (4,16). Confirmation of cloned viral DNAs was subsequently carried out with restriction enzymes analysis.

Nucleic acid spot hybridization test

Isolation of total DNA from pigeonpea plant — Total genomic DNA was isolated from susceptible, moderately susceptible, resistant and symptomless plants of mungbean and pigonpea. One gram of young trifoliate leaves was ground with pestle and mortar in 1.5 ml of extraction buffer as described earlier. Total DNA was isolated and dissolved in 100 μl of sterile double distilled water (17).

Preparation of radio-labeled DNA probes — Full length viral DNA inserts were released by digesting DNA-A and DNA-B clones with *Pst* I and *Bgl* I. The viral DNA fragments were separated by 1% low melting point agarose gel electrophoresis running overnight at 8 volts. The derived full length 2.7 kb MYMIV DNAs were cut out from the gel and then denatured by keeping boiling in waterbath for 10 min. The α-^{32}P was incorporated by random priming method (18) using labeling kits (Amersham). The incorporation of label was measured for each probe in a scintillation counter (Packard Tri-Card 1600 TR) for preparing probes of equal strength.

Nucleic acid spot hybridization — DNA samples of 5μg (dilution 1), and 0.5 μg (dilution 1/10) DNA were dotted on Nylone membrane (Hybond N, Amersham). Blots were baked at 80°C for 2 h. Each blot was sealed in a polythene bag containing 3 ml of prehybridization solution (6x SSC, 5x Denhardts reagent, 0.3% SDS, 100 μg ml^{-1} denatured fragmented Salmon sperm DNA) and incubated at 65°C for 18 h. To each blot, probe of 3.5 x 10^6 dpm was added and then incubated further at 65°C for 18h. Blots were washed twice at room temperature for 5 min and thrice for stringent wash at 65°C for 15 min in the wash buffer (2x SSC, 0.1% SDS). Blots were exposed to X-ray film (INDU QX 16) and the films were developed as per the manufacturer's protocol.

Results

Molecular cloning of MYMIV-Mg DNA — Agarose gel electro-phoresis analysis showed that seven (from 12th to 18th fractions) out of 20 CsCl fractions from bottom end, contained viral DNA with two DNA bands, one open circular at upper position of lane corresponding to 3.0 kb and another supercoiled at lower position corresponding to 1.6 kb; these two bands were shown by one (16th fraction from bottom end) representative fraction (Fig.1a, lane 1). It also showed that the viral DNA migrated faster than the host DNA.

Replicative double stranded DNA of MYMIV Mg has unique *Pst* I site, as a linear band of the viral genome about 2.7 kb length was visualized on restriction analysis (Fig.1a, lane 3). A total of 30 transformed colonies of *E. coli* were obtained in IXA transformation plate. All these colonies were picked up for plasmid miniprep preparation. The recombinant DNAs were digested with *Pst* I and all of them released about 2.7 kb linearized viral DNA insert which was co-migrated with vector DNA band.

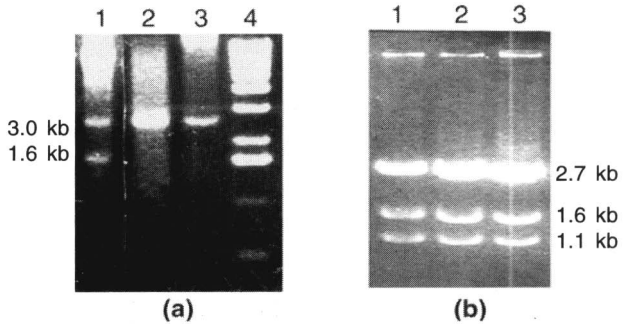

Fig. 1. (a) Gel electrophoresis of MYMIV DNA by CsCl density gradient centrifugation fractions. Lane 1: showing the lower 1.6 kb band of viral double stranded supercoiled form and upper 3.0 kb band of linear replicative form; lanes 2 and 3: linearized pUC 18 vector and MYMIV DNA form digested with *Pst* I, respectively and lane 4: 1 kb DNA marker, and **(b)** Gel electrophoresis showing 2.7 kb MYMIV-Mg insert from two populations; Lane1: showing 2.7 kb MYMIV-Bg DNA-B cloned earlier digested with *Bam* HI/*Bgl* I, lanes 2 and 3: 2.7 kb DNA from pMYMg 14 and pMYMg 31 digested with *Pst* I/*Bgl* I, respectively.

Double digestion of recombinant plasmid DNAs with *Pst* I and *Bgl* I released a insert size of about 2.7 kb (1.6 kb + 1.1kb) (Fig. 1b) length which is the anticipated full length genome of begomoviruses. The preliminary restriction analysis of these 30 recombinant plasmid DNAs have shown two categories of distinct DNA types. One of the types (19 out of 30) had unique site for *Eco* RI and *Kpn* I and other types had not. These two DNA types were considered to represent DNA-A and DNA-B of the MYMIV Mg. Two clones, one each from the two DNA types, designated as pMYMg 14 and pMYMg 31, respectively were selected. The clones, pMYMg 14 and pMYMg 31

were identified as DNA-A (2746 bp) and DNA-B (2670 bp) components after complete nucleotide sequencing (accession numbers, DNA-A: AF 416742 and DNA-B: AF 416741). As DNA-A component of this MYMIV-Mg showed 94% identity with other MYMIV isolates, the isolate was considered as variant of the species of MYMIV and hereafter, described as MYMIV-Mg isolate.

Use of MYMIV DNAs as probe for detection of MYMIV — Full length [α- ^{32}P] radio-labeled DNA-A and DNA-B of MYMIV-Mg were used as probes in NASH. Both the probes could successfully detect MYMIV DNAs in infected mungbean genotypes PS 16, TAM 9201 and MGG 443, and pigeonpea genotypes H 86-14 and MUA-1 (Fig. 2a, b). Hybridization reaction showed that concentration of DNA-A is less than DNA-B in all the infected genotypes of mungbean, whereas, concentrations of both the DNAs were found to be similar in infected pigeonpea genotypes.

Identification of pigeonpea genotypes resistant to MYMIV and detection of symptomless infection — Most of the pigeonpea genotypes produced visible symptoms within 9-27 days after inoculation in greenhouse condition (data shown only against MYMIV-Pp1). The time taken to produce symptoms in resistant and susceptible genotypes did not vary much. The different genotypes reacted differently and produced symptoms differing in disease severity; the disease reaction ranged from resistance to susceptible when inoculated with the variants of MYMIV in greenhouse

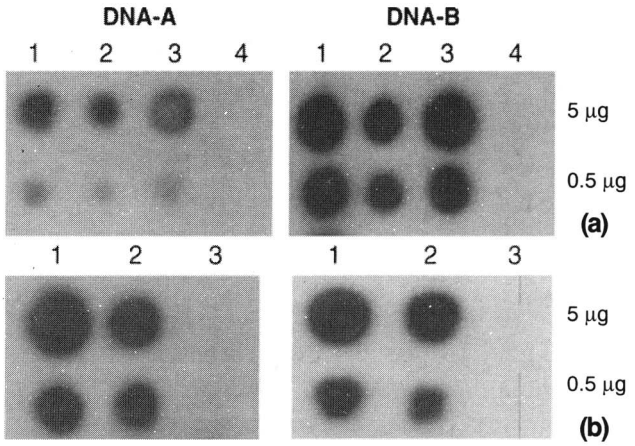

Fig. 2. Detection of naturally occurring MYMIV infection in mungbean and pigeonpea plants by NASH with [α-^{32}P] labeled MYMIV-Mg DNA-A and DNA-B full length probe. **(a)** Lane 1: susceptible mungbean genotype PS 16, lane 2: moderately susceptible genotype TAM 9201, lane 3: resistant genotype MGG 443 and lane 4: healthy control from un-inoculated genotype PS 16, and **(b)** Lane 1: susceptible pigeonpea genotype H 86-14, lane 2: resistant genotype MUA-1 and lane 3: healthy control from un-inoculated genotype H86-14.

condition (results not shown). All the pigeonpea genotypes tested were found to be infected by MYMIV-Pp1. Of these, six genotypes were highly resistant, three were resistant and others were susceptible (Table 1). Nine pigeonpea genotypes were inoculated with variant MYMIV-Bg. Of these, two genotypes, H 82-1 and ICPL- 84023 produced very mild symptoms (resistant) and other seven genotypes

Table 1. Infection and severity of pigeonpea genotypes against different variants of MYMIV

Genotype	No. of plants infected/ No. of plants inoculated	Days taken to develop symptoms	Disease severity of MYMV against variant[a]				
			Pp1	Bg	MgD	MoL	Mg
H 82-1	4/14	12-27	1	1	3	2	2
H 86-1	3/7	27	1	0+	2	2	2
H 86-14	4/8	13-21	3	0+	2	-	1
ICPL-1	4/8	12	3	0+	-	2	-
ICPL-83015	2/5	12-27	3	1	3	2	2
ICPL-84023	6/21	12-17	2	1	0+	0+	1
MUA-1	4/16	13	1	-	1	1	2
Pusa 605	9/18	9-14	1	-	1	2	2
Pusa 609	4/9	9-16	1	0+	3	2	2
Pusa 610	4/7	12-27	2	-	3	0+	1
Pusa 611	7/15	10-28	1	1	-	2	3
Pusa 856	3/16	12	2	0+	1	3	1
Pusa 860	3/7	12-27	3	0	2	0+	3
TAT-14	14/17	11-26	3	0+	3	0	0

[a]: Disease severity on 0-3 scale. +: viral DNA detected by NASH; - : not tested

did not produce symptoms (Table 1). These seven genotypes were examined by NASH using DNA-B probe to determine the presence of viral DNA. Six genotypes, H 86-1, H 86-14, ICPL-1, Pusa 609, Pusa 856 and TAT-14 showed positive hybridization reaction (Fig. 3, lanes 2, 3, 4, 7, 9 and 12). One genotype i.e. Pusa 860 was found to be immune as no hybridization signal was observed in NASH (Lane 10). Eleven genotypes of pigeonpea were tested for their disease reaction to MYMIV-MgD, of which three genotypes, MUA 1, Pusa 605 and Pusa 856 were highly resistant (Table 1). Only one genotype, ICPL-84023 was found to be symptomless. However, in this genotype viral DNA was detected by NASH (Fig. 3, lane 5). Other genotypes were found to be susceptible.

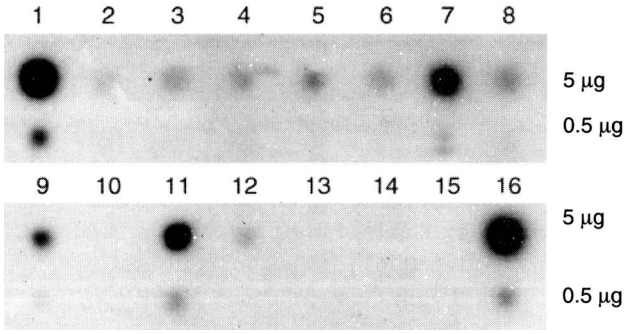

Fig. 3. Detection of MYMIV infection in different symptomless genotypes of pigeonpea against different variants of MYMIV by NASH using α-^{32}P la beled MYMIV-Mg DNA-B full length probe. Lanes 1 and 16: positive control from genotype TAT 14 infected with MMIV-Pp1, lane 2: H 86-1 with Bg, lane 3: H 86-14 with Bg, lane 4: ICPL-1 with Bg, lane 5: ICPL 84023 with MbD, lane 6: ICPL-84023 with MoL, lane 7: Pusa 609 with Bg, lane 8: Pusa 610 with MoL, lane 9: Pusa 856 with Bg, lane 10: Pusa 860 with Bg, lane 11: Pusa 860 with MoL, lane 12: TAT-14 with Bg, lane 13: TAT-14 with MoL, lane 14: TAT-14 with MbS, and lane 15: healthy control from un-inoculated genotype TAT-14.

Against variant MYMIV-MoL, four pigeonpea genotypes ICPL-84023, Pusa 610, Pusa 860 and TAT-14 did not produce symptoms, however except TAT-14 all the three symptomless genotypes showed positive reaction in NASH (Fig. 3, lanes 6, 8 and 11) and genotype TAT-14 did not show any reaction to MYMIV-MoL (immune reaction) also no DNA was detected using NASH (Fig. 3; lane 13). Only one genotype MUA-1 was highly resistant, seven genotypes were resistant and one genotype, Pusa 856 was susceptible (Table 1). Of the 13 pigeonpea genotypes tested against variant MYMIV-Mg, one genotype TAT-14 did not produce symptoms, four genotypes were highly resistant, six genotypes resistant and two genotypes, Pusa 611 and Pusa 860 were susceptible (Table 1). TAT-14 was proved to be immune as no MYMIV DNA was detected by NASH (Fig. 3, lane 14).

Discussion

Full length inserts of MYMIV-Mg DNAs indicate that genome of MYMIV consists of two distinct DNA components of about 2.7 kb designated as DNA-A and DNA-B. The bipartite nature of MYMIV-Mg DNA resembles the Indian MYMIV-Bg isolate (4) and most of the other whitefly transmitted gemeiniviruses (1,4,19).

Radiolabeled probes prepared from MYMIV-Mg DNA-A and DNA-B successfully detect MYMIV infection in different plants. Detection of yellow mosaic viruses by homologous as well as heterologous DNA-A and DNA-B probes of MYMIV and *Indian cassava mosaic virus* (ICMV) was demonstrated earlier by nucleic acid hybridization (1,19). Although both the MYMIV-Mg DNA-A and DNA-B probes successfully detected MYMIV infection, use of MYMIV DNA-B as a probe would be more specific to detect the MYMIV infection in plants.

Naturally occurring MYMIV variants caused varied degrees of disease severity in pigeonpea genotypes. In the present study, no pigeonpea genotype was found to be immune or symptomless against MYMIV-Pp1 suggesting it as a most virulent variant of MYMIV. The variant MYMIV-Pp1 has also been reported earlier as the most virulent on blackgram and mungbean plants (10, 11).

The genotypes ICPL-84023, MUA-1 and Pusa 605 would be most promising pigeonpea genotypes because these were either resistant or highly resistant to all the MYMIV variants used in the present study. The genotype, TAT-14 has been found to be immune to MYMIV variants MoL and Mg, though symptomless infection was obtained with the variant Bg. From the results obtained in the present study, it is hypothesized that resistance to pigeonpea is specific to a particular variant or strain of viruses. It is the first evidence of identification of resistance in pigeonpea against different variants of MYMIV.It is also the first evidence of symptomless infections of MYMIV in pigeonpea. Incidence of symptomless infection varies depending on variants prevalent in natural condition. Some variants can cause maximum symptomless infection. Symptomless infection caused by MYMIV in other leguminous crops like blackgram and mungbean has also been reported earlier (10,11,14).

The genotypes of pigeonpea identified as resistant to MYMIV will be very useful for breeding for resistance to MYMIV in this crop. Earlier no effort has been made to examine resistance in pigeonpea to MYMIV as it was considered as a minor problem. However, infection of the crop with different variants of MYMIV and occurrence of symptomless infection in many genotypes have indicated the potential of pigeonpea acting as a major reservoir of MYMIV for susceptible leguminous crops, source of initial inoculation particularly in the areas where the pigeonpea crop is maintained by ratooning . Therefore, there is an urgent need to evaluate resistance or immunity to MYMIV in the commercial genotypes so that only the resistant genotypes are recommended for cultivation.

Interestingly, in NASH test, both in symptomless or resistant genotypes and in susceptible genotypes of pigeonpea, virus titre of same magnitude was obtained. It shows that virus multiplication and its DNA accumulation are not inhibited in retesitant or symptomless genotypes but other molecular events leading to symptom expression may be affected.

The present investigation thus, suggests that many genotypes of pigeonpea are infected by MYMIV with no visible symptoms. It is highly probable that symptomless plants serve as reservoir of acquisition and transmission of virus to susceptible crop (15). The easy and sensitive diagnostic methods as developed in the present study based on DNA probes of cloned MYMIV, would be a useful tool for the detection of MYMIV symptomless infection in pigeonpea genotypes. This would help in breeding pigeonpea for resistance to MYMIV.

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