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Search for *Vigna* species conferring resistance to *Mungbean yellow mosaic virus* in mungbean

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Abstract

Mungbean yellow mosaic virus (MYMV) is a disastrous pathogen of mungbean. It is widespread in most of southern India and no complete resistance has been identified among its commercial cultivars. Two isolates of MYMV, representing its diversity, were used to assess and characterize the susceptibility reaction of all the three species of *Vigna*. The seeds were agroinoculated with the virus and the presence of the viral DNA was confirmed after 12 d by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis after which the plants were monitored for the expression of symptoms. All of the 20 accessions of *Vigna radiata* and ten accessions of *Vigna mungo* were systemically infected with MYMV, and they all produced typical symptoms. On the other hand, the 24 accessions of *Vigna umbellata* were found to be resistant to both the isolates. For additional affirmation, three representative accessions of *V. radiata* and *V. mungo* and all the accessions of *V. umbellata* were agroinoculated, and quantitative RT-PCR was performed for the quantitative detection of the MYMV. The mRNA transcripts of MYMV were detected in *V. radiata* and *V. mungo* plants but not in the *V. umbellata* plants. Researching the molecular basis of the resistance in V. umbellate against MYMV might definitely be very constructive for developing resistant varieties of mungbean on a commercial scale. This genetic quality offering resistance to MYMV could also be incorporated into *V. radiata*/*V. mungo* by means of interspecific crosses.

Keywords: agroinoculation; *Mungbean yellow mosaic virus*; qRT-PCR; resistance sources; screening; *Vigna* species

Introduction

Mungbean yellow mosaic virus (MYMV) is one of the most destructive viral diseases of mungbean (*Vigna radiata* (L.) Wilczek). It causes severe yield losses and reduces the quality of seeds. In India, MYMV affects all mungbean-producing regions of the country and is the prime viral disease of the state of Tamil Nadu (Usharani *et al*., 2004). The isolates of MYMV have been classified into two distinct strains. The reassortants between those two strains are grouped based on their nucleotide sequences (Balaji *et al*., 2004). Only a few research groups are committed for commercially resistant mungbean cultivars against MYMV (Shanmugasundaram *et al*., 2010). Asian vegetable research development center (AVRDC) has released two resistant lines, namely, NM 92 and NM 94 (which is resistant

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to MYMV during the summer but is susceptible during the June–July sowing). Resistance is based on an increase in the disease influx, whitefly populations and unfixed levels of resistance (Nair *et al.*, 2013). Hence, the hunt for new resistant lines became a laboriously continuing process. Some transgenic research had been attempted but with no fruitful results. So, the introgression of unexplored wild genes into cultivated varieties could be rewarding in broadening the genetic base for resistance (Monika *et al.*, 2001; Bisht *et al.*, 2005; Pandiyan *et al.*, 2008; Naimuddin *et al.*, 2011). This would be an effective and economic method in the control of the virus in mungbean (Karthikeyan *et al.*, 2012). MYMV is transmitted by a vector, the whitefly (*Bemisia tabaci*) in a persistent (circulative) manner. The efficacy of transmission and the behaviour of whiteflies suggested that there is a gamut of susceptibility reactions for each strain of MYMV for every host it infects on. The reaction on infection was so unique for each strain and exclusively diverse. It varied with the genotype of the host, the biotype of the vector and the growth conditions. Based on these conspicuous infectious patterns, researchers kept on trying and a simpler molecular tool called agroinoculation was identified to study the plant–virus interactions (Vaghchhipawala *et al.*, 2011; Bi *et al.*, 2010). It could be very useful in screening the resistant germplasms in pulse-breeding programmes (Biswas and Varma, 2001; Karthikeyan *et al.*, 2011). The tumour-inducing plasmid (containing the DNA sequence that produces tumour on infection) of *Agrobacterium tumefacians*, a common soil bacterium, is used as an alternate route for introducing the infectious viral (strains) clones into the plants. The infection results in the release of a genome-size copy of viral DNA into the infected cells of the host, which replicates and encapsidates, and systemically spreads, and the expected disease symptoms are expressed on time. Thus, agroinoculation in the test genotypes produces a more uniform disease epidemic condition than the natural infestation. The uniformity is significant and easier to compare among diseased genotypes. The primary objective of this research is to identify genetic sources of resistance from wild species of *Vigna* against MYMV and screening for the most resistant line among *V. radiata*, *Vigna mungo* and *Vigna umbellata*, by agroinoculation with the two viral isolates.

Materials and methods

Plant materials

A total of 20 accessions of *V. radiata* and ten accessions of *V. mungo* and 24 accessions of *V. umbellata* (Table S1, available online) originated from different parts of India were acquired from the National Pulse Research Centre (NPRC) located near Pudukkottai in the state of Tamil Nadu in India. The accessions used for this study were selected based on the field-screening studies conducted previously by Pandiyan (2004).

Isolates of MYMV

The infected leaves of MYMV from the fields of *V. mungo* in the village, Vamban of the Pudukkottai District in Tamil Nadu, were collected by Balaji *et al.* (2004). They constructed the infectious clones, namely, VA 221 (KA30 DNA A + KA22 DNA B) and VA 239 (KA30 DNA A+ KA27 DNA B) by cloning the MYMV genomes from the infected leaves of *V. mungo*. The infectious clones were mobilized in the strains of *A. tumefaciens*, namely, *Ach* 5 and *C* 58. The two mobilized strains of MYMV (VA 221 and VA 239) were obtained from Balaji *et al.* (2004) and were used for our screening studies in the accessions of *Vigna*.

Agroinoculation

Agroinoculation was done on sprouted seeds of 54 accessions of *Vigna* (20 accessions of *V. radiata*, 24 accessions of *V. umbellata* accessions and ten accessions of *V. mungo*) that were 2 d old (Jacob *et al.*, 2003). The agroinoculated plants were maintained in a growth chamber at 25°C with 60–70% of relative humidity for a photoperiod of 16/18 h. The Hoagland's solution was applied twice a week for proper growth of the plants. The development of symptoms in the trifoliate leaves was recorded from the 7th day from inoculation. The presence of yellow mosaic symptoms on the plants in a given time is considered as its susceptibility and the absence of it is scored as its resistance against the disease. The percentage of infectivity in the mungbean plants through agroinoculation is calculated based on the number of infected plants to the portion of the number of plants inoculated. After 12 d from agroinoculation, the leaf samples were collected from all the agroinoculated plants, frozen in liquid nitrogen and stored at −80°C immediately.

Extraction of total RNA

Total RNA was extracted using an RNA Kit, Madison, WI 53711, USA (Promega, USA) following the manufacturer's instructions. The quality of the RNA was checked in 1.5% agarose gel by visualizing the intactness of the RNA bands in agarose gel electrophoresis and the

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quantification of the RNA was done by the NanoDrop Spectrophotometer, Thermo Scientific, Inc. Wilmington, DE 19810, USA (Thermo Scientific, Inc.).

Conventional reverse transcriptase-polymerase chain reaction assay

Conventional reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the gene-specific primers (forward primers – 5′-GCGGAATTACG-ATACCGCC-3′, reverse primers – 5′-GATGCATGAGTAC-ATGCC-3′) of the coat protein to confirm the presence of the viral DNA. The primers were designed by the software, Primer 3, Premier Biosoft International, Palo Alto, CA, based on the Coat protein (*CP*) gene sequences of the isolates of MYMV of Tamil Nadu (Vamban MYMV isolates that were found in the National center for biotechnology information (NCBI) database). The extracted RNA was converted into complementary DNA (cDNA) using RevertAid™ H Minus First Strand, Fermentas cat no. R0081, Thermo Scientific, Inc. Wilmington, DE 19810, USA. cDNA Synthesis Kit (Fermentas cat no. R0081). The first strand (cDNA) was used as a template for the synthesis of the second strand through RT-PCR with the primers of the coat protein of MYMV. The temperature cycles were programmed as follows: 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The final elongation step was extended to 5 min at 72°C and finally maintained at 4°C. The PCR products were detected in 2% agarose gel in Tris/Borate/EDTA or (Tris base, boric acid and EDTA) (TBE) buffer and visualized by ethidium bromide (0.5 μg/ml) staining.

Quantitative real-time PCR

Three representative accessions from *V. radiata* and *V. mungo* and all the accessions of *V. umbellata* were selected for agroinoculation (with the strains, VA 221 and VA 239) (Jacob *et al.*, 2003), and the levels of mRNA transcripts were studied. To know the multiplication of virus in these plants, the leaves were collected from plants after 15, 20, 25, 30 and 60 d from inoculation (at least three replications with six individuals each) and the total RNA was extracted and converted into cDNA (as described above). The quantitative PCR (qPCR) reaction for each sample was performed using the ReverTra Ace® qPCR RT Kit, Osaka 530-8230, Japan (TOYOBO, Japan) and a Bio-Rad iQ™5 system (following the manufacturer's instructions). The qRT-PCR mixture contained 8 ml of diluted cDNA, 10 ml of 2× iQ™ SYBR® Green Supermix Bio-Rad, Singapore, 609924 (Bio-Rad, Singapore), and 200 nM of gene-specific primers (mentioned above), and was finally made up to a volume of 20 ml. The control for the PCR (without cDNA templates) was also performed for the aforementioned primers. The reactions were run for 2 min at 94°C, followed by 35 cycles of 94°C for 60 s and 60°C for 30 s on a 96-well optical reaction plate (Bio-Rad iQ™5 system, Singapore, 609924). The levels of mRNA were measured via qPCR using SYBR® Premix Ex Taq™ (Bio-Rad, Singapore). After that, the fluorescence was measured through the melting curve analysis at 55–94°C to detect a gene-specific peak and the relative copy numbers of *CP* genes were calculated using the $2^{-\Delta\Delta C_t}$ method.

Results

Screening of the species of Vigna *against MYMV through agroinoculation*

Two distinctive MYMV strains, VA 221 and VA 239, were used to typify the reactions to MYMV on infection, in 54 accessions of *Vigna* through agroinoculation. Subsequent to agroinoculation, confirmatory studies using RT-PCR were carried out in all the inoculated and control plants (12-d-old leaves) to provide evidence for the presence of the viral DNA inside the host genome. Oligonucleotide primers that are specific to the gene of coat protein of DNA A of MYMV (of amplicon size 703 bp) were used

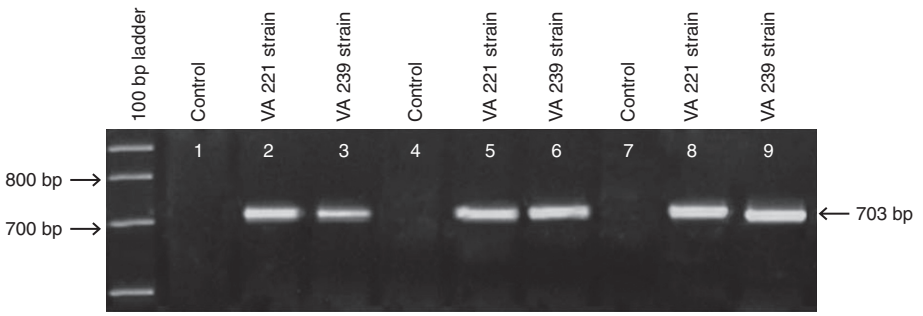

Fig. 1. Amplification of DNA A of coat protein gene of MYMV by RT-PCR in the agroinoculated plants (12th day). Lanes 1–3: *V. mungo* – CO 5; lanes 4–6: *V. radiata* – VRM (Gg) 1; lanes 7–9: *V. umbellata* – TNAU RED.

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for this purpose. The viral DNA was detected only in the inoculated plants and was absent in the control (Fig. 1). Furthermore, the development of symptoms in the trifoliate leaves was recorded after 7 d from inoculation. The 54 accessions of *Vigna* showed different responses due to infection (the presence of MYMV resulted in the development of typical viral symptoms). All the 20 accessions of *V. radiata* and ten accessions of V. mungo exhibited yellow mosaic symptoms in the trifoliate leaves upon agroinoculation, for both the strains VA 221 and VA 239 (Fig. 2). The infection percentage varied from 10 to 100% (data not shown). The agroinoculated plants started developing yellow mosaic symptoms from the 13th to the 25th day, and there were no symptoms in the control. However, accessions of *V. umbellata* were not systemically infected with the two distinctive strains of MYMV. Interestingly, all of them were found to exhibit higher levels of resistance. The ricebean plants did not develop mosaic symptoms even after 2 months from inoculation (Fig. 2). All the results were found to be the same in all the replications and the percentage of infectivity was null (0%). To corroborate the results, the agroinoculation experiment was repeated again for confirmation and the very same results were obtained.

Detection of the virus in the infected plants on different stages of plant growth (in day after inoculation)

Three representative accessions from *V. radiata* and *V. mungo* and all the accessions of *V. umbellata* were selected for agroinoculation (with the strains, VA 221 and VA 239). To understand the process of infection by MYMV and to register the levels of mRNA transcripts on infection by MYMV in the plants, a qPCR method was adopted. It was helpful to detect the relative levels of mRNA transcripts in the inoculated plants on the 15th, the 20th, the 25th, the 30th and the 60th day from

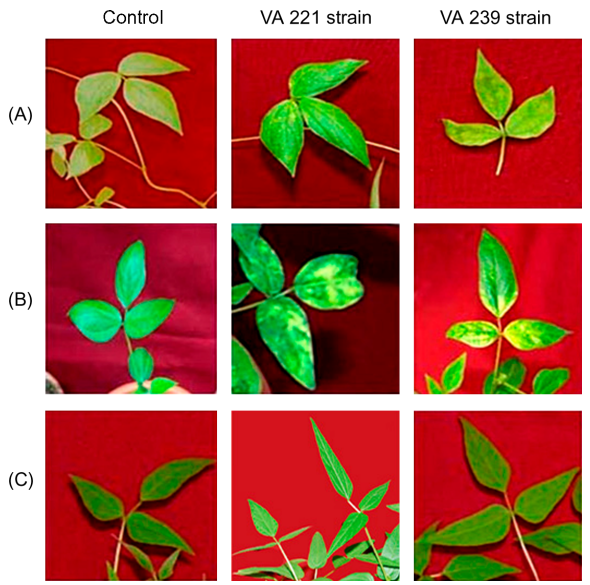

Fig. 2. Development of symptoms of MYMV in the agroinoculated plants (A) *V. mungo* – CO 5 (B) *V. radiata* – VRM (Gg) 1 and (C) *V. umbellata* – TNAU RED.

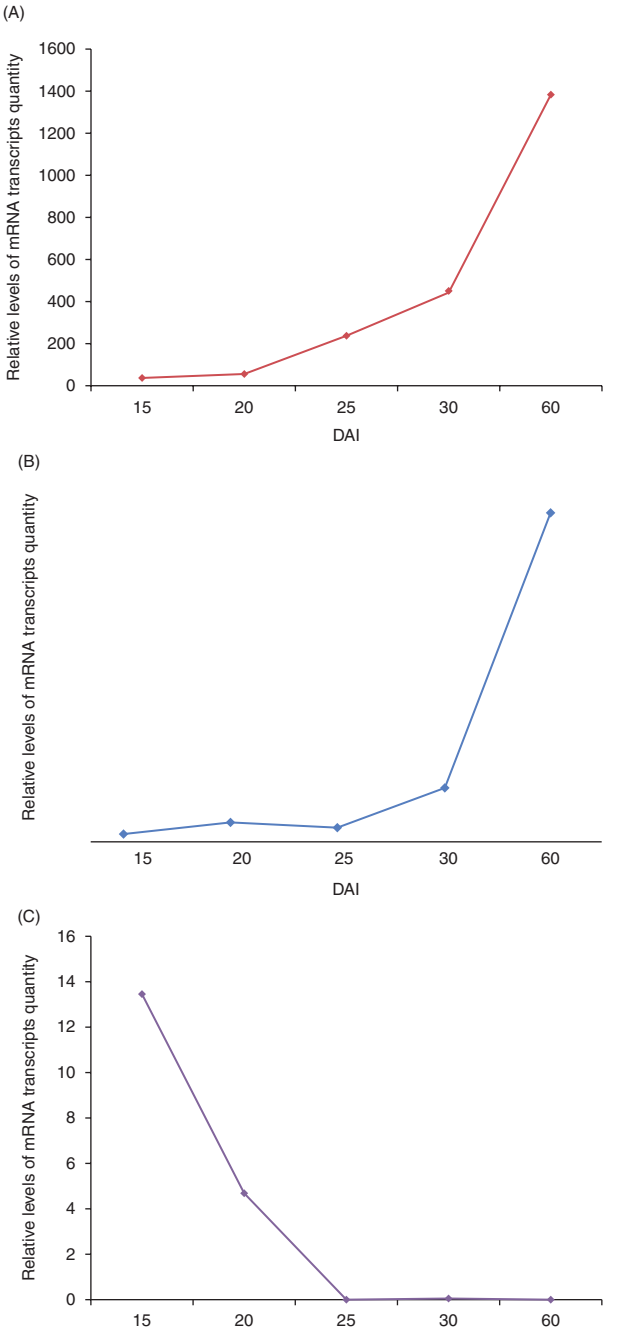

Fig. 3. Relative levels of mRNA transcripts of MYMV *CP* gene in (A) *V. mungo* – CO 5, (B) *V. radiata* – VRM (Gg) 1, (C) *V. umbellata* – TNAU RED.

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inoculation (Fig. 3) (all the data are not shown here). In the accessions of *V. radiata* and *V. mungo*, the levels of mRNA transcripts were relatively high on the 15th day and it started increasing rapidly from the 15th to the 20th day. However, the levels of the mRNA transcripts were high on the 25th, the 30th and the 60th day. At the same time, the results observed on the accessions of *V. umbellata* were so interesting, in which different levels of mRNA transcripts were detected in the leaf samples collected on the 15th and the 20th day after inoculation (DAI). The levels of mRNA transcripts decreased from day 15 to day 20. There was no mRNA transcript detected on the rest of the days (25th, 30th and 60th DAI).

Discussion

In this study, 54 *Vigna* accessions, obtained from NPRC, were screened for resistance to MYMV infection using agroinoculation and the presence of viral DNA was confirmed by RT-PCR amplification of the 703 bp region of the *CP* gene of MYMV. The expression of symptoms was seen in all the accessions of *V. radiata and V. mungo*, but not in any accessions of *V. umbellata*. Furthermore, we investigated the level of mRNA transcripts of MYMV by the SYBR® Green I-based real-time quantitative PCR, Bio-Rad, Singapore, 609924. The levels of mRNA transcripts of MYMV were detected on the accessions of *V. radiata* and *V. mungo* using the real-time PCR on all the days (15, 24, 30 and 60 DAI). At different intervals, the level of mRNA transcripts was high. It showed the highest peak on the 60th DAI in both the species. At the same time, the results found in the accessions of *V. umbellata* are the key for understanding the mechanism behind the resistance for MYMV. The mRNA transcripts of the coat protein gene were detected only from leaf samples collected on the 15th DAI and it was reduced on the 20th DAI. There were no mRNA transcripts during the rest of the days (25th, 30th and 60th DAI). The mRNA transcripts of the viral genome until the 20th day indicated clearly that the multiplication and accumulation of virus had happened inside the cell but some unknown mechanism had prevented the further multiplication of the virus. So, it was absent in the plant during later inoculations. Interestingly in the accessions of *V. umbellate,* MYMV was detected on 15 and 20 DAI (confirmed with the real-time PCR). But there was no visible symptom of MYMV observed in the *V. umbellata* plants. At the same time, the plants of *V. radiata* and *V. mungo* showed the typical symptoms. It is possible that some tissue-specific host tolerance mechanism is operating inside the plant, resulting in this sort of resistance reaction. Differences in the host range of yellow mosaic virus isolates have been studied by various workers, but the results continue to be ambiguous (Varma and Malathi, 2003). The difficulty was mainly due to the feeding behaviour of the vector, the host genotypes used and the environmental conditions. This was mainly attributed to the less permissive nature of the ricebean hosts because, otherwise, the clones are highly infectious on other legumes after agroinoculation. These evidences are very much supportive to the statement that ricebean is a host and is resistant to MYMV. However, comprehensive molecular investigations at the protein level should be conducted, allowing researchers to understand the resistant mechanism of ricebean against MYMV. In mungbean breeding programmes, screening, identification and evaluation of systemic resistance in the source material is critical due to the narrow genetic variability in the primary gene pools. This is due to the limited gene pool of the cultivated species of *Vigna*. For producing the crop types, which combine quality, higher productivity and resistance to disease and pest, it is necessary to widen the gene pools of the cultivated species through interspecific hybridization. Varying degrees of success in interspecific hybridization of *Vigna* have been reported successful, particularly the cross between *V. radiata* × *V. umbellate* (Bharathi *et al.*, 2006; Pandiyan *et al.*, 2010). In this study, complete resistance to MYMV has not been identified in *V. radiata*. But, 24 accessions of *V. umbellata* are proved to be highly resistant, which could provide an elite array of resistant source for effective breeding of mungbean cultivars against MYMV.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262114000859

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