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RCA-PCR Assay is More Effective Than Traditional PCR Assay for Detecting Begomoviruses in Greengram

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Abstract Rapid and accurate detection of the causal virus is a prerequisite for monitoring plant virus epidemics and specific diagnosis of causal virus associated with disease is critical for disease management and for control of virus spread through vectors. There is a need to develop a reliable and effective technique to detect a causal virus. In the present study nine symptomless greengram samples were subjected to traditional PCR with virus specific primers to DNA-A and DNA-B of Mungbean yellow mosaic virus and DNA-A of Mungbean yellow mosaic India virus. Out of total nine, three samples were showed positive results with Mungbean yellow mosaic virus DNA-A specific primers and two samples were amplified with Mungbean yellow mosaic virus DNA-B specific primers similarly five samples were found positive with Mungbean yellow mosaic India virus DNA-A specific primers. The samples which were shown negative in PCR assay again subjected to RCA-PCR assay for further confirmation. In RCA-PCR assay all nine samples were found positive with DNA-A and DNA-B of Mungbean yellow mosaic virus specific primers and DNA-A Mungbean yellow mosaic India virus specific primers. Hence it is proved that RCA-PCR assay is more sensitive than traditional PCR assay to detect Begomovirus.

Keywords Begomovirus, Detection, Rolling circle amplification, Greengram, Polymerase Chain Reaction.

Introduction

Greengram is a nutritious grain legume crop containing 23.6% of easily digestible protein and 51% carbohydrates. It is cultivated in China, Thailand, the Philippines, Vietnam, Indonesia, Myanmar, Bangladesh, India, and in the hot and dry regions of Southern Europe and Southern United States [1]. Combined yield losses due to Begomoviruses were estimated to exceed $300 million in blackgram, greengram, and soybean in India [2]. One of the most destructive and important viral diseases of mungbean and urdbean is yellow mosaic disease (YMD). The disease mainly occurs in South Asian countries, viz. India, Sri Lanka, Pakistan and Bangladesh. The disease can reduce mungbean seed yield by up to 100% or even kill a plant infected at an early vegetative stage. YMD is caused by geminivirus (genus Begomovirus, family Geminiviridae), which has bipartite genomes (DNAA and DNA B). Begomoviruses cause Mungbean yellow mosaic virus (MYMV), Mungbean yellow mosaic India virus (MYMIV), Dolichos yellow mosaic virus (Do YMV) and Horsegram yellow mosaic virus (HgYMV), which have spread throughout South Asia. MYMV and MYIMV are the main pathogens caus-

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ing YMD in mungbean grown in India [3]. The prevailing techniques for geminivirus detection include biological indexing, electron microscopy, ISEM, antibody-based detection, including ELISA, dot blot nucleic acid hybridizations and polymerase chain reaction using either specific or degenerate primers [4] associated with YMD of greengram and blackgram. The high serological variability of the virus is also likely to give false-negatives during serological identification using procedures such as the enzyme linked immuno-sorbent assay (ELISA). Therefore robust technique for detection of these viruses is a prerequisite for their effective management. However, fast evolving characteristics of the single-stranded viral genomes lead to failure of detection of these viruses through these techniques and even detection of these virus in ultra low viral titre where PCR fails, is a critical for future management. To overcome these problems, many laboratories employ a rolling circle amplification (RCA) technique which in fact revolutionized the diagnosis of geminiviral infection. This technique has the advantage of amplifying any circular DNA without the initial knowledge of the sequence and it is better, easier and cheaper than polymerase chain reaction (PCR) or antibody based detection [5]. In this study we compared the detection levels of RCA-PCR assay along with traditional PCR assays with virus specific primer.

Materials and Methods

Total DNA extraction

Nine symptomless greengram samples were collected from the fields of Regional Agricultural Research Station, Tirupati total DNA were isolated by modified CTAB method at their seedling stage. The DNA samples were quantified using Nanodrop spectrophotometer (ND1000, USA).

Rolling circle amplification

Total DNA from greengram leaf samples at seedling stage was used as the template for RCA, which was conducted using *phi*-29 DNA polymerase. Briefly, 20-50 ng of total DNA was mixed with *phi*-29 enzyme buffer, dNTPs (1 mM), exo-random hexamers (50 IM), and denatured for 3 min at 95^0C. After cooling the reaction mix to room temperature, 0.02 U of pyrophosphatase and 6-8 U of *phi*-29 DNA polymerase were added and incubated at 30^0C for 18-20 h. During this process, the random hexamers anneal to single-stranded circular DNA templates at multiple sites. *phi*-29 DNA polymerase then extends each of these primers unitil the DNA polymerase reaches a downstream-extended primer, at which time strand dis-placement synthesis occurs. As the process continues, exponential isothermal amplification occurs. After thic incubation, the reaction was stopped for 10 min at 65^0C [6].

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in 25µl of reaction mixture using 1× PCR reaction buffer, 2.5mM of $MgCl_2$, 10 mM of each dNTPs, 10 pmols of each primer, 2.5 U/µl of Taq DNA polymerase (Fermentas, USA) and 100ng of DNA template. The same reaction was carried out with 100ng of rolling cirele amplification (RCA) product. The amplification was performed in a PCR machine (Eppendorf Pro S, Germany) and the conditions for amplification of coat protein gene and other primers were studied and their annealing temperatures are given in Table 1. The am-

Table 1. Specific primers used for amplifying selected regions of DNA-A and DNA-B of MYMV; DNA-A of MYMIV DNA-A.

Primer name	Nucleotide sequence (5'-^{2}')	Target molecule	Amplicon size	Annealing temperature
MYMV-CP	F-TGGGATCCATTGGTGAACGA R-TACGCACGACCTGATAACGA	Partial DNA-A	700bp	59^0C
MYMV-MP	F-ATGGAGAATTATTCAGGCGCA R-TTACAACGCTTTGTTCACATT	Partial DNA-B	900bp	55^0C
MYMIV-CP	F-GGTCCCCTGATGTCCCTCGTG R-ATGCGTTCTCAGTATGGTTCT	Partial DNA-A	500bp	55^0C

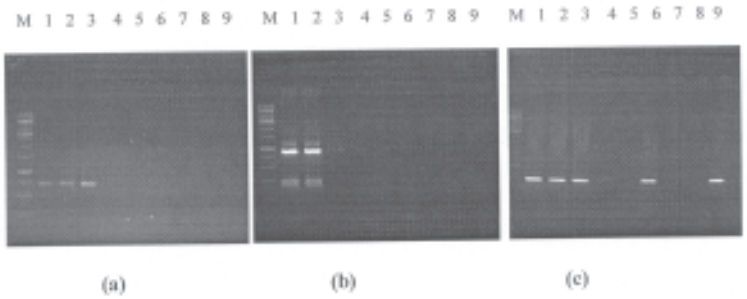

Fig. 1. PCR amplification of greengram samples by using Mungbean yellow mosaic virus coat protein primers (a), Mungbean yellow mosaic virus movement protein primers (b) and Mungbean yellow mosaic India virus coat protein primers (c). Lane M: 1Kb ladder Lane 1-9: different greengram samples.

plified PCR products were separated on 1% agarose gel in 1x TBE buffer at 100 V. The banding pattern was documentated in gel documentation system (Alpha Innotech, USA).

Results and Discussion

Out of total nine DNA samples, three samples were shown amplification with MYMV-CP primer (Fig. 1a), two samples were found positive with MYMV-MP primer (Fig. 1b) and five samples were shown positive results with MYMIV DNA-A primer (Fig.1c) by PCR. The remaining negative samples were tested using RCA-PCR assay for further confirmation along with positive samples confirmed by traditional PCR. In RCA-PCR, all nine samples showed the amplification of an expected product size with respective primers (Fig. 2). Failure to detect the virus in PCR is due to low concentration of the viral DNA in the total plant DNA which is insufficient for amplification using PCR.

Detection of the begomovirus associated with greengram and blackgram using dot blot nucleic acid hybridizations, ISEM and polymerase chain reaction with specific or degenerate primers techniques have been reported so far. PCR was found more sensitive than NASH for the detection of new world begomoviruses. However, the amplification may be very faint or absent in samples having very low virus titre [7]. Begomovirus could detect in a higher percentage of samples using RCA-PCR than PER [8].

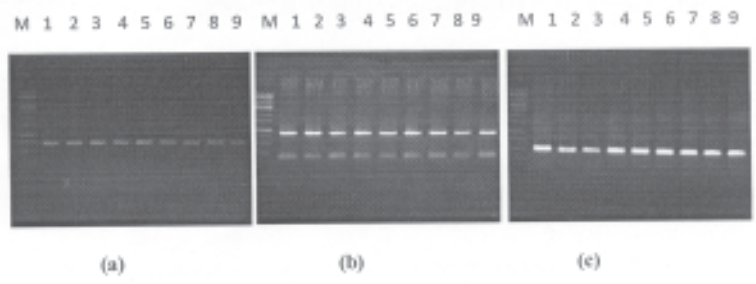

Fig. 2. RCA-PCR amplification of greengram samples by using Mungbean yellow mosaic virus coat protein primers (a), Mungbean yellow mosaic virus movement protein primers (b) and Mungbean yellow mosaic India virus coat protein primers (c). Lane M : 1Kb ladder Lane 1-9: different greengram samples.

The same results with ToLCNDV-potato reported that the RCA-PCR is 10^9 times more sensitive than PCR assay [9]. This confirms that RCA reaction before PCR assay leads to better sensitivity than PCR alone and the higher sensitivity of the RCA-PCR assay may be due to the amplification of circular viral DNA to many folds by RCA before the PCR assay.

Conclusion

The yellow mosaic disease of greengram and blackgram was known in India since 1960, but the causal virus could not be identified due to paucity of the detection tools at lower virus titre. This study optimized RCA-PCR assay to detect Begomoviruses in symptomless samples with very low virus titre, which could be used as reliable diagnostic tool to identify the MYMV and MYMIV, an emerging Begomoviruses in India, not only in greengram and blackgram but in other hosts like soyabean, cowpea and pigeonpea that might be infected by this newly spreading virus in future. Hence, RCA-PCR assay is more effective and sensitive method to detect Begomovirus compared to traditional PCR assay.

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