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Full Length Research Paper

Rapid and sensitive detection of potyvirus infecting tropical tuber crops using genus specific primers and probes

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A reverse transcription polymerase chain reaction assay using potyvirus specific primers designed from the core of the coat protein was carried out, and a cDNA fragment of 327 bp was obtained from most of the potyviruses infecting the tropical tuber crops. Reverse transcription polymerase chain reaction (RT-PCR) products were sequenced and found to be derived from the expected virus. Specific cDNA probe was generated from the amplicon, and the probe was then successfully used for the diagnosis of the potyviruses infecting the major tuber crops through biotinylated nucleic acid spot hybridisation. The specific probe developed could detect the potyviruses infecting tuber crops namely SPFMV, DsMV and DAV from sweet potato, aroids and yams respectively.

Key words: Coat protein, potyvirus, reverse transcription polymerase chain reaction, nucleic acid spot hybridisation.

INTRODUCTION

Tuber crops form the means of sustenance for millions of people in the tropical and subtropical world. The world's total harvested area of tubers is nearly 51 million hectares with one third found in Africa and one third in Asia and Pacific regions. Tuber crops are generally rain fed in nature, and these store starch in their vegetative parts, mostly inside roots or subterranean stem modifications (tuber, corms, stolons). Tuber crops have a higher biological efficiency as food producers and show the highest rate of dry matter production per day per unit area among all the crops. These crops are recognized as the most efficient converters of solar energy. Apart from that these tuber crops are known to supply cheap source of energy especially for the weaker sections of the population. These attributes make tuber crops ideal for cultivation in the less developed countries of the world. Among the tropical tuber crops of economic importance, cassava (*Manihot esculenta*), *sweet potato* (*Ipomoea batatas*), yams (*Dioscorea* species) and aroids namely taro (*Colocasia esculenta*), tannia (*Xanthosoma sagittifolium*) and elephant foot yam (*Amorphophallus paeoniifolius)* are the major tropical tuber crops cultivated in many countries including India. These tubers are popularly used as vegetables in various delicious cuisines, possess medicinal properties and also serve as by-products for industries.

The genus *Potyvirus* (in the family *Potyviridae*) contains the largest number of plant virus species among 34 plant virus groups and families currently recognized including 91 formal species and 88 tentative species (van Regenmortel et al., 2000). Potyviruses cause significant losses in a wide range of crop plants and are transmitted by aphids in a non-persistent manner. They cause significant losses in agricultural, pasture, horticultural and ornamental crops (Shukla and Ward, 1991). Tropical tuber crops are no exception. The entire aforementioned tuber crops are known to be infected by one or the other species of potyvirus. Cassava brown streak virus (*Ipomovirus* in *Potyviridae* family) in cassava, *sweet potato feathery mottle virus* (SPFMV) in sweet potato,

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Dioscorea alata virus (DAV) and *yam mosaic virus* (YMV) in yams and *Dasheen mosaic virus* (DsMV) in edible aroids are the major potyviruses causing economic loss in tuber crops. Virions of potyviruses are flexuous filaments, 680 to 900 nm long and 11 to 13 nm wide. The single-stranded, positive-sense RNA genome of potyviruses (ca. 10 kb) is polyadenylated at the 3' end and has a viral genome-linked protein (VPg) covalently linked to the 5' end. The whole genome encodes a single polyprotein subsequently processed into 9 to 10 proteins by three virus-encoded proteinases (van Regenmortel et al., 2000). Earlier the serological diagnostic methods like Enzyme linked Immunosorbent Assay (ELISA) have been successfully used for the large scale detection and diagnosis of plant viral diseases (Clark and Adams, 1977; Flegg and Clark, 1979). But the serological method of ELISA possess major limitations such as high cost, extensive labour, higher duration and low sensitivity outside the plants' vegetative period because of the low titre values of the pathogens. Moreover serological diagnosis of potyviruses is often imprecise, because of frequent serological cross-reactions between species (Brunt, 1992) and biological indexing is very cumbersome. With the advances in the field of molecular biology, nucleic acid-based methods such as reverse transcription (RT) and the polymerase chain reaction (PCR) began to be used in plant virus detection (Hsu et al., 2005; Wetzel et al., 1991; Rowhani et al., 1995; Thomson and Dietzgen, 1995).

The available potyvirus sequences in the database made possible the development of a method for the identification of potyviruses based upon the polymerase chain reaction (PCR) (Langeveld et al., 1991). For PCR based identification of the potyvirus group, local conserved regions in the core domain of the potyvirus coat protein were selected for the construction of degenerate primers for application in a potyvirus group-specific combined assay of reverse transcription polymerase chain reaction (RT-PCR). Accordingly, several degenerated primers have been designed to recognize the conserved regions of viral genomes of many virus species or the whole virus genus or family (Langeveld et al., 1991; Bateson and Dale, 1995; Tian et al., 1996; Gibbs and Mackenzie, 1997; Chen et al., 2001; Posthuma et al., 2002). Combining the RT-PCR technique and degenerated primers, it is possible to detect many virus species of the same genus or family. Another strategy for the identification of potyviruses is through the development of species-specific probes, a method similar to gene or mRNA detection using microarrays (Gerhold et al., 1999; Harrington et al., 2000; Kane et al., 2000; Lockhart and Winzeler, 2000).

The aim of this study was to work out a uniform, sensitive and potyvirus group specific primers based RT-PCR and nucleic acid spot hybridization using group specific probe for the detection of all the major potyviruses infecting tuber crops. Except cassava, where there is no report of cassava brown streak virus (CBSV) in India, the protocol described here could detect potyviruses infecting all other tropical tuber crops in India. In this study, a detection method which can examine whether a tuber crop plant is infected by potyviruses and an identification method which is capable of identifying species of potyvirus infecting tuber crops were developed. A potyvirus group specific probe was also developed and a uniform protocol for detection of potyviruses infecting the entire tropical tuber crops using this probe has also been standardized and validated with the leaf samples collected from field.

MATERIALS AND METHODS

Virus sources and RNA isolation

Five different species of potyvirus were used in this study: *Dasheen mosaic virus* isolates from *Amorphophallus paeoniifolius, Xanthosoma sagitifolium* and *Colocasia esculenta, sweet potato feathery mottle virus* from sweet potato*, Dioscorea alata virus* from *Dioscorea alata,* maintained at glass house of CTCRI were used. Cassava plants infected with cassava mosaic virus was also checked since there is no occurrence of CBSV or other potyvirus on cassava in India. Varying symptomatic leaf samples of these plants were collected separately, and total RNA (approximately 65 µg/µl) was extracted from 100 mg of these plants using the QIAGEN RNeasy plant mini kit (Maryland, USA) according to the manufacturers' protocol and resuspended in 50 µl nuclease free water.

RT-PCR

The isolated RNA from the different samples was subjected to single step RT-PCR using one step RT-PCR kit (Finnzymes, Espoo, Finland). The RT and PCR reactions were performed in a single 25 µl PCR tube with the reaction mixture (20 µl) containing 10 ng RNA, 1 µl of 20 pmol oligo $d(T)_{16}$, 1 µl of 20 pmol each of potyvirus group specific primers, MJ1-5'-TGGTHTGGTGYATHGARAAYGG-3' and MJ2-5'-TGCTGCKGCYTTCATYTG- 3', (Marie-Jeanne et al., 2000; Grisoni et al., 2006), 2.0 µL of 10x Reaction buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 50 mM magnesium chloride ($MgCl_2$), 0.25 µl of AMV Reverse Transcriptase and 1.0 µl of Dynazyme DNA polymerase (1 U/ µl). The RT-PCR was performed in Eppendorf Mastercycler Gradient ES with the following thermal programme: Reverse Transcription at 48°C for 45 min, initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min and a final extension of 72°C for 10 min. The amplified product was analyzed on 1% agarose gel, stained with ethidium bromide and photographed under UV-gel doc system (Alpha imager). The PCR product was purified using the QIA quick PCR purification kit and the product was then subjected to automated sequencing. The sequenced strands were then edited using the BIOEDIT Software (Hall, 1999). The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/) with Blastn.

Probe preparation

The biotinylated DNA probe containing the 327 bp partial coat protein gene of the DsMV, amplified from *A. paeoniifolius*

was prepared employing the technique of random primer Biotin labelling of DNA using the NEBlot^R Phototope kit (New England Biolabs). The process was carried out as per the standard protocol: Gel eluted template DNA was diluted to a total volume of 34 µl and denatured at 100°C for 5 min and immediately chilled on ice. The solution was centrifuged briefly and the following components were added: 10 µl of 5x labeling mix, 5 µl of dNTP mix, 1 µl of Klenow fragment, and the mixture was incubated at 37°C for 2 to 3 h and the reaction terminated by adding 0.2 M EDTA (pH 8.0). The probe was precipitated by incubating at -20°C (1 h) with the addition of 4M $LiCl_2$ and 150 µl of ethanol. The mix was centrifuged briefly, washed with 70% ethanol and resuspended in 20 µl 1x TE. Serial dilutions (10^{-1} to 10^{-6}) of the biotinylated probe and the prebiotinylated markers in 0.1 N NaOH were analysed for checking the probe quality.

Hybridisation using DNA probe

Total RNA (5 µl each) from the different plants (approximately 20 ng of each samples) were directly spotted on to the Nitrocellulose membrane in 1 cm^2 squares and UV cross linked. The membrane was subjected to prehybridisation for 1 h at 68°C (20x SSC, 5x Denhardts reagent, 0.5% SDS, Denatured Salmon Sperm DNA), followed by hybridization (prehybridisation solution containing 20 ng/ml denatured biotin labeled DNA probe) for overnight at 68°C. The membranes were washed twice with low stringency solution (2 x SSC, 0.1% SDS) followed by high stringency solution at 68°C (0.1 x SSC, 0.1% SDS). The membrane was incubated in blocking solution, followed by incubation in streptavidin (final concentration of 1 mg/ml). The membrane was washed twice in wash solution 1 (blocking solution 1:10 dilution), incubated in biotinylated alkaline phosphatase (1:1000 dilution in blocking solution), followed by a single wash in wash solution 1 and twice in wash solution 2. The membrane was then subjected to detection reagent 1x CDP star and exposed to X-ray film development.

RESULTS

Specificity of potyvirus degenerate primers

Total RNA were extracted from each virus-infected plant and analyzed by RT-PCR with potyvirus degenerate primers, MJ1 and MJ2. RT-PCR analysis of the total RNA isolated from different potyvirus infected tuber crops resulted in the amplification of 327 bp amplicon (Figure 1). Sequence analysis of the amplicons from the infected tuber crops confirmed the association of the corresponding potyvirus species (data not shown). Among the tuber crops, the potyvirus infecting elephant foot yam, taro and tannia was found to be the species DsMV of the genus *Potyvirus*. Among the randomly selected symptomatic leaves of five plants, the primers MJ1 and MJ2 showed a consistent amplification of 327 bp in all the samples of elephant foot yam and taro. However, in case of tannia, four plants showed a consis-tent amplification of 327 bp except one. Among the sweet potato leaf samples, all of the five samples showed an amplification of 327 bp, and sequencing and BLAST analysis confirmed the presence of *sweet potato feathery mottle virus*. Among the greater yam plants, four of the samples showed the amplification except one. There was no amplification in case of all the cassava samples tested indicating the absence of any potyviral infection.

Specificity of cDNA probe

The 327 bp amplicon from the elephant foot yam was used for the preparation of DNA probes. This cDNA probe was tested for identifying different potyviruses from tuber crops in a single test. The RNA samples from different plants were first immobilised on to the nitrocellulose membrane and were probed using the cDNA probe. The probe recognized correctly all of the viruses tested, including DsMV, SPFMV and DAV, the major potyviruses infecting tuber crops (Figure 2). The results of the nucleic acid spot hybridisation technique correlated with the results of the RT-PCR. All the samples except one each from tannia and greater yam, showed a positive result.

DISCUSSION

In order to evaluate the specificity of potyvirus degenerate primers, three viruses belonging to different species of potyvirus family infecting the tuber crops were used in RT-PCR tests. These primers (MJ1-MJ2) correspond to the conserved sequences located in the core of the CP regions, designed to amplify a short fragment spanning conserved motifs MVWCIEN to QMKAAA in the core of the CP of potyviruses. The primers were selected from among other previously published degenerate primers (Langeveld et al., 1991; Pappu et al., 1993; Chen et al., 2001) because they amplify a short fragment and they gave superior amplification signals in preliminary experiments. Moreover, analysis of these sequences with various potyviruses deposited in the NCBI database showed that the region is highly conserved (data not shown). The coat protein region of the potyvirus was used because it was considered to be the second most important region to cytoplasmic inclusion protein (CI) gene for species identification, with a demarcation criteria of 76 to 77% (Adams et al., 2005). Secondly the presence of many conserved motifs in the viral CP region facilitates the design of numerous degenerate primers for RT-PCR based detection of the virus genome (Langeveld et al., 1991; Pappu et al., 1993; Colinet et al., 1998; Marie-Jeanne et al., 2000; Chen et al., 2001). RT-PCR analysis of the samples using species specific primers MJ1 and MJ2 detected almost all the potyviruses infecting tropical tuber crops. However, RT-PCR analysis of one sample each of tannia and *C. esculenta* showed no amplification, even though the samples exhibited slight mosaic symptoms as similar to DsMV. The presence of the 327 bp amplification showed that the primer binding sites are conserved in most of the tuber crops infecting potyviruses and hence could be used as a rapid and sensitive

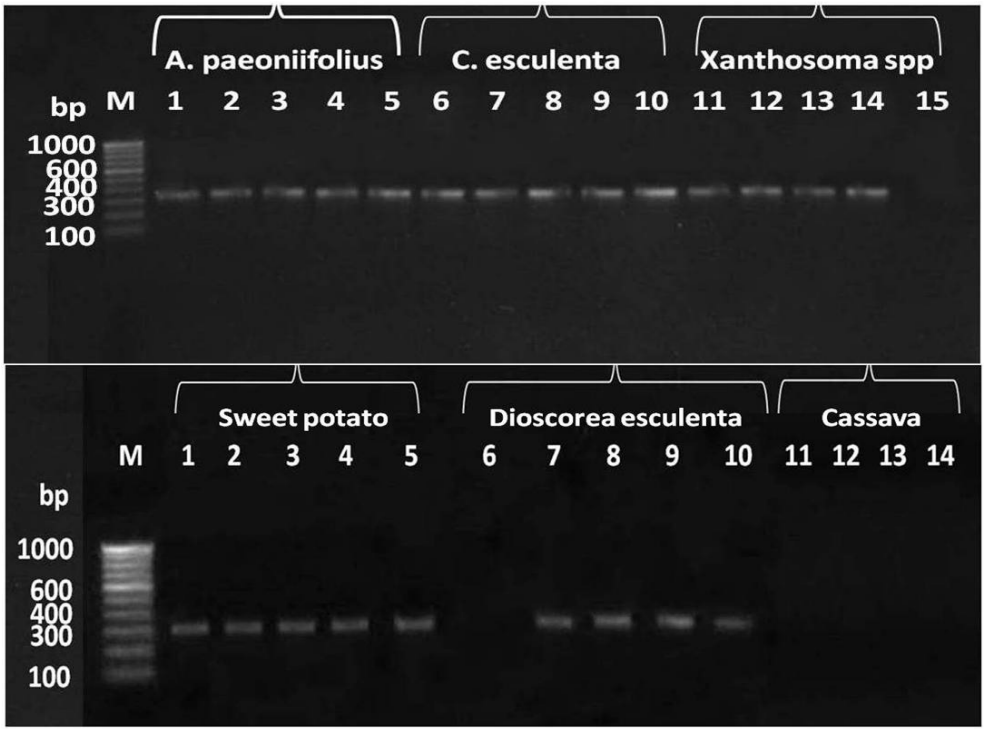

Figure 1. RT-PCR based detection of potyviruses infecting various tuber crops using genus specific primers.

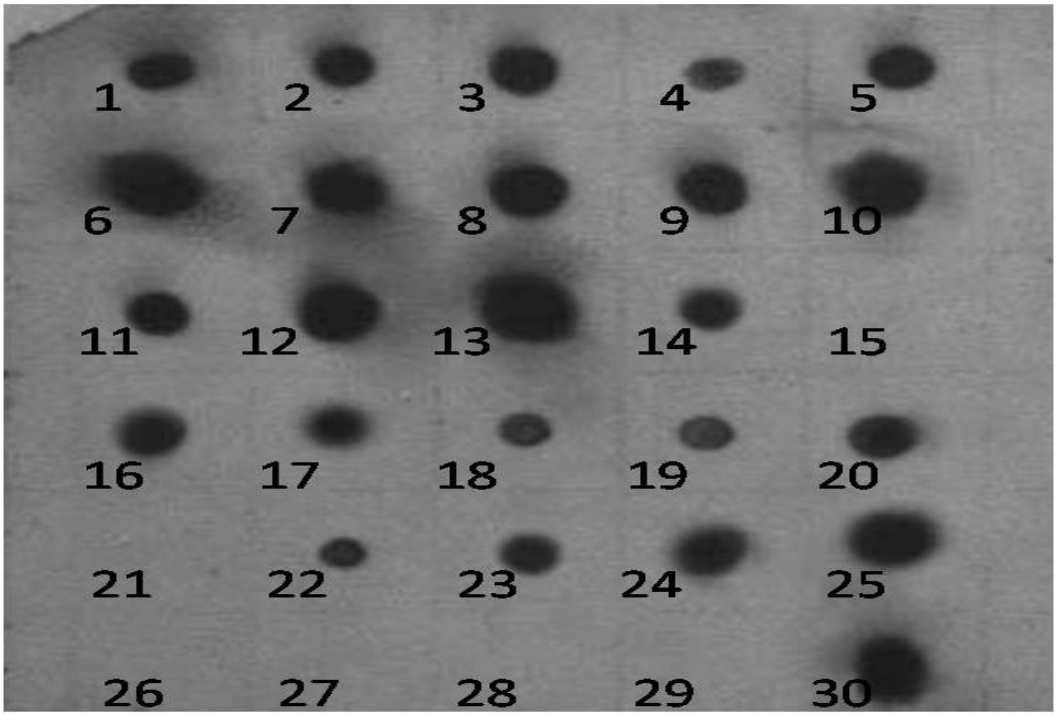

Figure 2. Detection of potyviruses infecting various tuber crops using genus specifc DNA probe. Lane 1-5: *Amorphophallus paeoniifolius* samples; Lane 6-10: *Colocasia esculenta* samples; Lane 11-15: *Xanthosoma sagittifolium* samples; Lane 16-20: *Ipomoea batatas* samples; Lane 21-25: *Dioscorea esculenta* samples; Lane 26-29: *Manihot esculenta* samples; Lane 30: Positive control (cloned plasmid containing 327 bp amplicon).

method for the detection of potyviruses infecting in tuber crops. Moreover the specific probe developed based on the conserved region of the potyviruses was able to detect the potyviruses under study, thus facilitating the easy and early identification of almost all the potyviruses infecting tuber crops.

Therefore, these primers could be readily used as a source for identifying species of potyviruses occurring in these tuber crops. Moreover, a highly specific probe developed from the 327 bp amplicon of the conserved potyviral region could facilitate the rapid and sensitive detection of potyviruses infecting tuber crops. Moreover, large scale indexing of the tuber crops for the presence of potyviral infection can be easily carried out with the specific primers and probes. Even though RT-PCR with the many specific degenerate primers could possibly detect most of the potyviruses, a disadvantage of the process is the difficulty in identifying the viral species infecting the plant, which requires further sequencing and sequence analysis. Since the RT-PCR with MJ1 and MJ2 primers produce an amplicon of shorter fragment (327 bp), it would again facilitate the further processing of the samples; for species identification. Thus, the potyvirus group specific primers used in this study and the probe developed is highly useful in the diagnosis of the potyvirus infecting the tuber crops and in the development of a complete identification chip (blot) for potyviruses which will speed up the identification procedure for potyviruses in infected tuber crop plants.

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