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Coat Protein Gene: A key tool for *Yam Mild Mosaic Virus* diagnosis in greater yam

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Abstract

Destruction of infected plants and use of virus-free planting materials are the common control measures used for preventing viral infections. These practices are inadequate to combat the virus attack in many incidents. Early detection of infection is an effective way to manage the systemic primary spread of viruses. Infection caused by *Yam mild mosaic virus* (YMMV), one of the major viruses in yams (*Dioscorea* spp) is difficult to detect in the early stages. Symptoms include mild mosaic, leaf mottling, and chlorosis and leaf distortion. In the present study, PCR was employed for the amplification of YMMV coat protein (CP) gene for diagnosing the virus from greater yam (*Dioscorea alata* L.) leaves. The specific primer pair was designed and validated for CP gene amplification, which yielded an amplicon of 810 bp in YMMV positive samples. Subsequent cloning in pUC18 vector and sequencing confirmed the presence of the full coat protein (CP). In addition to PCR-based diagnostic method, the accomplished isolation and characterization process opens avenues for generating virus-specific polyclonal antibodies through the utilization of the expressed coat protein. These antibodies can be further employed in serological techniques.

Keywords: YMMV, *Dioscorea alata,* PCR, Diagnosis, CP primer, cloning

Introduction

Yam is a common name for several species of *Dioscorea* which are tuberous starchy food cultivated and consumed in developing countries. They ranked as the third most important tuber crop after cassava and sweet potato (Fu et al., 2005). The most important edible yams are *D. alata*, *D. rotundata*, *D. esculenta* and *D.bulbifera*. Yams are essential for many tropical and subtropical livelihoods (Cao et al., 2021).The principal edible yams are cultivated mainly in three different regions such as Asia, Africa and South America and also the temperate regions (Lebot, 2009). They are vegetatively propagated crop and the species are characterized by weak climbing stem and underground tubers or rhizomes but some species produce aerial tubers too. These tubers are source of carbohydrate for millions of people around the world although some species are of medicinal and ornamental value (Padhan et al., 2002). In India greater yam is the important species, which is cultivated and consumed largely in the Southern and North Eastern states. Among them large number of wild yam species are utilized in the state of Kerala, where a dozen of them are consumed by the people belong to tribal community in the Wayanad district, which contains parts of Western Ghats (Balakrishnan et al., 2003). Yam can be stored longer than other fresh products which results in increased commercial value.

The yam production is adversely affected by pathogenic diseases (Amrutha et al., 2022). Virus diseases are of

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particular importance because the reduced vigor results in tuber yield loss and quality (Kolychikhina et al., 2021; Bakayoko et al., 2021). The prominent viruses that infect yams belong to the genera of *Potyvirus, Badnavirus, Cucumovirus, Potexvirus and Macluravirus* (Diouf et al., 2022). In India the reports about the presence of such viruses is scanty. A survey conducted by ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI) revealed the presence of *Yam mild mosaic virus* (YMMV) and *Yam mottling virus* (YMoV) (CTCRI, 2009). Diseases caused by viruses are hard to be controlled by the use of chemical applications unlike those caused by fungi and bacteria even though some techniques like hot water treatment found applicable in virus elimination from younger nodes (Jayaseelan et al., 2011).

Use of infected planting materials is a major means of spread of yam viruses and may restrict the international movement of selected germplasm due to quarantine restrictions. There is a requirement for testing planting materials for ensuring the exchange of virus free germplasm and for disease control. Management of yam virus disease is mainly through the principle of exclusion by using healthy planting materials and prevention of virus infection through cultivation of virus resistant varieties (Sastry et al*.,* 2014). Thus knowledge on accurate diagnosis necessitates sustainable yam production. In this study, we used PCR for the early detection of YMMV infection employing specifically designed primers.

Materials and Methods

Collection of sample

For identification of YMMV positive samples, leaves showing mild mosaic, leaf mottling, leaf distortion and chlorosis were collected from greater yamgrowing fields at ICAR-CTCRI (Fig.1). Asymptomatic samples showed no prominent symptoms except some mild indistinct chlorosis were also collected. This representative sample set was used for further test and analysis.

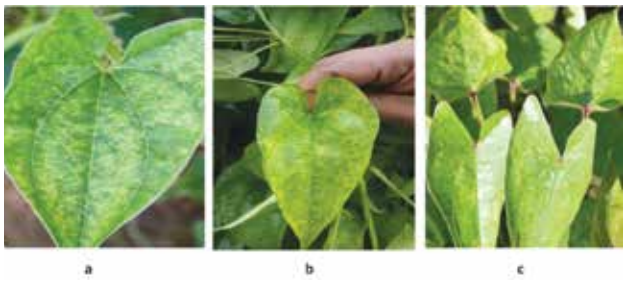

Fig. 1. Leaf samples showing mild mosaic (a), leaf mottling (b) and chlorosis (c)

RNA isolation and cDNA synthesis

Total RNA isolation was carried out using AmbionPurelink RNA Mini kit from the collected leaf samples of greater yam showing symptoms of various infection. The fully opened younger leaves were used. Younger yam leaves are most suitable than older leaves (Sika et al., 2015). The quantity and quality of the isolated RNA were measured on a NanoDrop spectrophotometer (DeNovix DS-11+ spectrophotometer) using RNase -free water as blank and performed an electrophoretic run on 1% agarose gel. Then visualized under UV to observe and document band for total RNA, typically show two major bands, corresponding to the 28S and 18S. This is because a reduced amount of tannins and phenolics in the younger leaves compared with the older leaves. Although the RNA yield obtained from leaf samples using AmbionPurelink RNA Mini kit method proved to be better method for good quality RNA.

Isolated RNA is less stable for long periods of storage; however, cDNA conversion ensures that the sample is not lost and was an essential prerequisite for conducting PCR based virus detection. From the isolated RNA, single-stranded cDNA was synthesized using Revert Aid FIRST strand cDNA synthesis kit (Thermo Scientific) with oligo dT primer. Approximately 2μg of total RNA was used in a 20 μL reaction with primers as described by the manufacturer's protocol. The reaction mix contain 4 μL of 5X Reaction Buffer, 1 μL of RiboLock RNase, Inhibitor (20 Uμ^{-1} L^{-1}), 2 μL of 10 mM dNTP Mix and 1 μL of RevertAid M-MuLV RT (200 Uμ^{-1} L^{-1}) and the volume was made up to 20 μL using Nuclease-free water. RNA was converted to cDNA using standard thermal conditions (single step reaction for 60 minutes at 42°C and the reaction were terminated by heating at 70°C for 5 min RNA was converted into cDNA for further PCR based screening. cDNA synthesis using Revert Aid FIRST strand cDNA synthesis kit was positive even from low quality RNA in most cases.

Amplification of partial coat protein gene

To identify YMMV positive samples, amplification of partial CP was performed using cDNA as a template and YMMV 1s 5'CACTCTTATGGTCTTGTT3'and YMMV 1c 5'TCTTATATGGTTCCTGTTC3' as forward and reverse primers (Sudheer, 2015), respectively. Amplification was done using (EmeraldAmp GT PCR Master Mix, Takara) in a thermocycler (BIO-RAD C1000 Touch) with initial denaturation of 4 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing 52°C for 01 minutes and extension temperature 72°C for 1 min and final extension of 10 min at 72°C. To visualize a single band, an electrophoretic run was performed on a 1.5% agarose gel, followed by UV visualization for observation and documentation.

Designing and validation of full coat protein primers

Specific primer pair was designed by multiple alignments of sequences of different isolates of *Yam mild mosaic virus* available in NCBI database. They were designed for the amplification of conserved coat protein coding region with restriction sites EcoRI (GAATTC) and HindIII

(AAGCTT) for the ease of cloning. The primer sequences were checked for various parameters including annealing temperature, AT and GC content, primer dimer formation, and self-complementarity. The annealing temperature of the specific primer was optimized by using gradient PCR (BIO-RAD C1000 Touch) from 50-60°C, and the conditions for PCR amplification was standardized.

Amplification of full coat protein gene

YMMV positive samples confirmed through partial coat protein amplification were collected from the greater yam fields of ICAR-CTCRI and total RNA was extracted from fresh young, infected leaf tissue (100 mg). Amplification was done using EmeraldAmp GT PCR Master Mix (Takara) in a thermocycler (BIO-RAD C1000 Touch) with initial denaturation of 4 min at 94°C followed by 35 cycles of denaturation at 94°C for 40 sec, annealing 54°C for 70 seconds and extension temperature 72°C for 1 min and final extension of 10 min at 72°C. After confirming the presence of specific amplicon, PCR products were purified by gel elution kit (Nucleo spin gel and PCR clean-up Macherey- Nagel) and the quality was confirmed for cloning.

Cloning

The amplicon and the plasmid pUC18, restriction digested with EcoRI and HindIII (New England Biolabs) were purified and ligated into pUC 18 cloning vector. A 20 μl ligation reaction mix is designed to ligate insert and vector DNA molecules to form a recombinant molecule. The overnight incubation at 16°C provides optimal conditions for the ligation reaction. The mix contained insert DNA, vector DNA, T4 DNA ligase reaction buffer, and 1 μl of 5 unit $\mu^{-1}l^{-1}$ T4 DNA ligase (Thermo Scientific).

Transformation

200 μl of competent DH5 alpha cells was added to the ligation reaction mixture in the vial, which was kept in ice for 30 min prior to heat shock at a temperature of 42°C for 45 sec. in water bath. Immediately the vial was quenched into ice. 1 ml of Luria Bertani medium was added to the vial. After one hour of shaking incubation at 37°C, 100 μl cell suspension was spread on Luria Bertani Agar (LBA) medium containing Ampicillin (50 mg ml^{-1}) as selection marker, X-gal (20 mg ml^{-1}) and IPTG (40 μg ml^{-1}) for blue/white screening of recombinant colonies. Single white colonies were picked up from the Petri plate. Success rate of transformation is confirmed by running a colony PCR and a plasmid isolation followed by restriction digestion. Positive clones were selected and sequenced.

Results and Discussion

After first stranded cDNA synthesis, the detection of YMMV infection in all the samples were carried out using

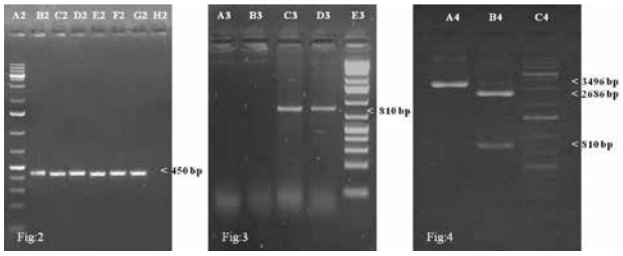

Fig. 2. (a) PCR amplification using YMMV lc and YMMV Is diagnostic primer yields a product size 450 bp (Lane B2-G2); (b) PCR amplification using forward Tcp(F) and reverse Tcp (R) primer to conform the full coat protein gene insert in the developed clone (Lane C3 & D3) showing 810 bp size PCR product marked against geneRuler 1kb plus DNA ladder (Lane E3) and (c) Gel image showing single digestion (LaneA4) of pUC 18 with insert, double digestion of pUC 18 (Lane B4) with 1 Kb pus ladder (Lane C4)

YMMV lc and YMMV ls specific primers, which amplifies the partial coat protein gene which yielded amplicons of 450 bp in samples positive for YMMV infection. There was no amplification observed in the non-template control which indicates that, no non- specific binding and primer dimer formation in PCR. The PCR results with YMMV lc and YMMV Is primers are shown in Fig. 2. The sequence results were initially analyzed and edited using BioEdit Sequence Alignment Editor Program version 7.2.5 and the obtained sequence was run through the online BLAST program of NCBI. The blast results query sequence of YMMV sequence showed maximum similarity 95% to *Yam mild mosaic virus* isolate DSMZ PV-1214 clone 1 polyprotein gene, (Accesssion no OM471977.1). A set of virus specific primers,Forward Tcp(F) primer 5'CCGAATTCGCAAGTAAGGAGCAG3' and Reverse Tcp(R) primer 5'GCAAGCTTGATATTACGCACTCC 3' which codes for the full length coat protein were designed and synthesized for the amplification of full CP gene of YMMV based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences. Primers were designed with restriction sites EcoRI and HindIII to ease the cloning procedure. The analysis of primers using program T_m Calculator (Thermo Scientific), revealed good GC content ideal annealing temperature, and also the designed primers did not exhibit hairpin formation and 3' complementarity. Performed a gradient PCR run with annealing temperature ranges from 50ºC to 60ºC to obtain the optimum annealing temperature and 54ºC for 70 sec. was the best for amplification. An amplicon of 810 bp was observed (Fig. 3) as a single band for virus positive samples previously diagnosed with primer YMMV lc and YMMV ls primer in 1.5 percentage agarose gel.

Viruses are of particular concern because, apart from causing significant reduction in tuber yield and quality, they restrict international exchange of germplasm. In this study, the samples were screened for their presence of

YMMV using nucleic acid based methods. PCR and RT-PCR techniques were employed to detect the presence of the virus.

In a preliminary study, screening to detect the virus was done using ELISA, it has major limitations such as its low sensitivity during periods of low titre. All leaf samples were also tested by PCR to ensure that plants with low virus load (Njukeng et al., 2002). In preliminary studies the samples screened through ELISA revealed that YMMV was the most common virus infecting greater yam. Similar observations of YMMV have been reported in *D. esculenta* from the Solomon Islands (Mumford and Seal, 1997). For confirmation of the presence of *Yam mild mosaic virus*, a published primer pair YMMV lc/YMMV 1s was used. PCR analysis with these primers yielded an expected product of size 450 bp. The newly designed TCP F/ TCPR in the study were used to amplify full CP gene (810 bp of) the virus from leaf samples of greater yam. The present investigation revealed the number of virus infections detected by PCR was more than that of the ELISA tests, possibly due to the high sensitivity of PCR. The lower sensitivity observed with the ELISA tests is similar to the findings of Mumford and Seal 1997 and could also be due to low virus concentration in yam (Brunt et al., 1990) or due to interference of polyphenols and glutinous polysaccharides contained in yam leaves (Rossel and Thottappilly, 1985). The development of primer targeting conserved regions in the genome is crucial for the detection of many viral variants (Davi et al., 2021). The transformed colonies harboring plasmids with the virus coat protein gene were analyzed for gene insert. Digestion of the plasmid with the restriction enzymes EcoRI and HindIII revealed a released insert of the expected size, 810 bp as seen in Fig. 4. The YMMV nucleotide sequence obtained in this studv showed maximum similarity of 98.03 % Yam mild mosaic virus isolate FIJI 3 polyprotein gene, (Accesssion AF548517.1) and 97.93% similarity to Yam mild mosaic virus Colombian isolate Col 2 polyprotein gene, partial cds (Accesssion AF548492.1).

Conclusion

Full coat protein gene of YMMV was amplified, sequenced, cloned and transformed for an Indian isolate which is related to the isolates from other countries. A PCR based diagnostic technique was standardized which is required for routine virus indexing and further the full CP amplicon could be utilized for developing viral coat protein leads to antibody production. This will prevent yield loss by the use of healthy planting material and safe exchange of germplasm.

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