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Original Research Article

Detection and analysis of leaf curl virus from Jatropha in north Indian region

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A B S T R A C T

Geminiviruses are a group of plant viruses that contain circular single stranded (ss) DNA genomes encapsidated in small twinned icosahedral capsids .The viral DNA A plus strand encodes the coat protein (CP/AV1) essential for viral transmission by whiteflies (*Bemisia tabaci*). Jatropha has been found to have strong molluscicidal activity and the latex to be strongly inhibitory to watermelon mosaic virus. It is also listed as a homicide, piscicide and raticide. Jatropha is considered as a poor quality fuel wood since the soft wood burns too rapidly. In Africa, Jatropha is widely planted as a "living fence" and hedgerows to protect food crops from damage by livestock and as a windbreak to prevent soil erosion moisture depletion. In Madagascar, Jatropha is used as a support plant for Vanilla. Jatropha is grown both as a vegetable and a spice crop. Out of the 22 viruses infecting jatropha, mosaic and leaf curl disease are the most devastating. Virus type--Single stranded DNA virus,Classification-Geminiviridae (Genus), Begomovirus (Family) .Viral composition--Has a monopartite viral genome size of 2,750bp. Also has a satellite molecule, DNA-β satellite with a genome of 1361bp.Disease symptoms-- Upward curling, vein clearing and reduced leaf size. Stunted growth and no fruit produced in severely infected plants. The virus is obligately transmitted by an insect vector, which can be the whitefly *Bemisia tabaci* or can be other whiteflies. This vector allows rapid and efficient propagation of the virus because it is an indiscriminate feeder. Natural conditions such as temperature of 25-35 Degrees C favor high vector population. Infected plant exhibits stunted young leaves and shoots. It grows very slowly, becomes bushy, and dwarfed. The leaf margin rolls either inward or upwards and is rather stiff with yellowish margin. Its leaves are thicker than normal, with leathery texture. The young leaves have yellowish color, cupped, thick, and rubbery. Isolate genomic DNA from jatropha plant from différent sample from diffrerent fields of lucknow. Then I have done quantitative and qualitative estimation of isolated DNA using nanodrop spectrophotometer and Agarose Gel Electrophoresis,respectively. Restriction digestion, PCR amplification of viral DNA with Begomovirus DNA-A specific primers, RAPD of a viral DNA.

Introduction

Jatropha (*Jatropha curcas* L.) also known as physic nut is a drought resistant perennial plant, which is popularly cultivated in the tropics as a living fence. The tree is of significant economic importance for its numerous industrial and medicinal uses. The oil extracted from Jatropha seeds is being used as biofuel for diesel engines thus jatropha has a great potential to contribute to the renewable energy source. In India the area under the cultivation of jatropha is increasing in recent years with the ever increasing demand for fossil fuels that are exhausting at a rapid rate.

Jatropha suffers from several fungal and bacterial diseases and more recently by the jatropha mosaic India virus (JMIV), which causes Jatropha mosaic disease (JMD) [2]. JMD was first reported from Karnataka state, South India in 2004 and was shown to be associated with a begomovirus based on virus transmission by the whitefly, Bemisia tabaci (Gennadius) and virus detection by polymerase chain reaction (PCR) tests [1]. However, the nature of the virus was unknown and its phylogenetic relationship with other begomoviruses was not established. Elsewhere, JMD was first reported on Jatropha from Puerto Rico and subsequently from Cuba and Jamaica. JMD in the Americas was shown to be associated with Jatropha mosaic virus (JMV), a bipartite begomovirus, which was also transmitted by *B. tabaci* in a semi persistent manner. [2]There is a growing intrest in Jatropha curcus as a biodiesel"miracle tree" to help alleviate the energycrisis and generate income in rural areas of developing countries. Jatropha oil is unclear how much genetics play in the amount of oil contained in

Jatropha seed and kernels; never the less, estimate of the oil content in seeds range from 35-40% oil and the kernels 55-60%[3].Jatropha nuts can be strung on grass and burned like candlenuts, and the oil to make candles. Unrefined Jatropha oil can only be used in certain types of diesel engines, such as Lister type engines. The glycerin that is a by-product of biodiesel can be used to make soap, and soap can be produced from Jatropha oil itself. The seed oil can be applied to treat eczema and skin diseases and to soothe rheumatic pain. The oil and aqueous extract from oil has potential as an insecticide. Jatropha oil is also used to soften leather and lubricate machinery. [4]Viruses of the genus *Begomovirus* (family *Geminivirida*) typically have bipartite, circular single-stranded DNA (ssDNA) genomes with all functions required for virus replication, control of gene expression and encapsidation encoded on DNA-A and genes involved in intra- and intercellular movement encoded on DNA-B[5] .

More recently many monopartite begomoviruses that have single DNA molecule are reported from bhendi, cotton and tomato [6]. These are associated with additional satellite molecules called DNA-β, which in some cases modulate symptom expression [7]. All begomoviruses encode a coat protein (CP) in which packages all the genomic and satellite molecules. The CP acts as the coat of the virus particle and is essential for virus transmission from diseased to healthy plants by *B. tabaci*. The CP is highly conserved amongst the begomoviruses originating from the same geographical region and thus been adapted to transmission by local vector populations [8]. The CP is therefore an essential component of begomovirus survival and has been used widely to characterise and establish the relationships of many begomoviruses . The core region of the CP sequences have also been used and shown useful for begomovirus diversity and classification purposes . In this study, JMIV was detected by PCR tests using two sets of begomovirus-specific degenerate primers. The core CP sequences were obtained and the phylogenetic relationship of JMIV with those of the American JMV and other begomoviruses was established. In addition, the JMD incidences, symptomatology, virus transmission by *B. tabaci* and a dodder parasitic plant have been demonstrated. Disease symptoms--Upward curling, vein clearing and reduced leaf size. Stunted growth and no fruit produced in severely infected plants.

Materials and Methods

Fresh leaves were collected from jatropha samples. Leaves were washed properly with distilled water and soaked them on the tissue paper. 150 mg of leaves were weighted and then add into DNA extraction buffer(1.5ml) and grinded them using pestle and mortar. We took 1ml of slurry in an ependorff tube. 66 µL of 20% SDS was added Extration buffer was vortexed and incubated for 15 min at 65 C. One third volume (354µL) of 5M potassium acetate was added in the extraction and then centrifuged at room temperature at 13,000 rpm for 15 min. 800µL supernatant was taken in fresh tube and then added equal volume of phenol and chloroform (400µL each tube). Then samples were centrifuged for 13,000 rpm for 15 min at room temperature. 650uL supernatant was taken in fresh ependorff tube and equal volume of chloroform and isoamyl alcohol (24:1) was added and then vertexed the samples.

These solution was centrifuged at 10,000 rpm for 5 min at room temperature. 500 µL of supernatant was taken in fresh tube. DNA was precipitated by adding 0.6 volume of ice cold isopropanol i.e,300 µL. precipitated DNA samples were centrifuged at 15,000 rpm for 15 min at room temperature. Supernatant were discarded. DNA pellets were washed with 200 µL 70% ethanol (ice cold). And pallets were centrifuged at 7000 rpm for 3 min at room temperature. Pallets were dried in dry bath at 50-55 C for 2 min. Resuspend the pellet in Pallets were resuspended in 30 uL double distilled water and were ncubated at 50 C for 5-8 min. Pallets were taped after 2-3 min. Quantification of the DNA was carried out by the nanodrom spectrometer. Isolated DNA was stored at -20 C for future use. 160 mg of agarose was weighed and dissolved into 20 ml 1X TAE buffer by heating with continuous swirling till a clear solution was obtained. To this solution, 2 µl of EtBr was added. The comb was placed into the casting tray. The use of comb depends on the volume of DNA to be loaded and the number of samples.

The molten agarose was poured on the gel casting platform with an inserted comb, ensuring that no air bubble have entrapped underneath the comb. After 30-45 minutes, when the gel sets/ hardens the comb was drawn taking care that the wells do not tear off. The gel was placed in electrophoresis tank. The gel tank was filled with sufficient volume of electrophoresis buffer i.e.,1XTAE buffer.For the PCR amplification TLCV primer was used, the following step was done.An initial denaturation step(94^{0}C,5 minute) was followed by 30 cycles of amplification (50 second at 94^{0}C.1 minute at 55^{0}C,and 1.5 minute at 72^{0}C).The temperature was maintained at 72^{0}C for 5 minutes after the last cycle. For RAPD-Setting up PCR, prepare a cocktail of PCR for 5 PCRs with five different target DNA samples. The variable components is to be added separated Sterile water,10X assay buffer,10Mm Dntp, Random primer, Taq DNA polymerase. Mix the content uniformly and gently.

All the above addition to be done on ice. Aliqout 20 µL of the above reaction mix to each of the five different PCR vials placed on ice and label the vials 1,2,3,4 and 5.Add 1 µL of serratia marcescenes genomic DNA to vial labeled 1.similarly add 1 µL each of genomic DNAs of Bacillus subtilis, E.coli B,E.coli K12,Test DNA Sample to the vials labeled 2,3,4 and 5 respectively. Mix the contents gently and overlay with 50 µL of mineral oil to prevent evaporation. Centrifuge the sample briefly (6000 rpm for 30 sec at 4^{o}C) to bring down the contents of the tube. Carry out the amplification using a thermocycler for 45 cycles aacording to the following condition. An initial denaturation step (95^{0}C at1 minute and denaturation 95^{0}C at 1 minute, annealing 45^{0}C at 1minute and extension 72^{0}C at 2 minute). The temperature was maintained at 72^{0}C for 5 minutes after the last cycle Following PCR amplification, add 5 µL of gel loading buffer to each of the PCR vials. Prepare 2% agarose gel. Mix the contents thoroughly and stands for few seconds for the two layers to separate. Carefully pipette out 15 µL of reaction mixture and load onto 2% agarose gel. Load 10 µL of the ready to use Low Range DNA ruler provided note down the order in which the samples are loaded. Run the sample at 100 volts for 25 to 30 min. till the tracking dye reaches the end of agarose gel. Visualize the gel under UV transilluminator.

Results and Discussion

The success of the isolation and extraction process of genomic DNA can be marked with resultant large DNA (high molecular weight DNA), that is not degraded during extraction and purification process, and can be cut by restriction enzymes that has been used.(FIG-1)To amplify the viral DNA from the DNA sample thus obtained, DNA-A specific primer was used.(FIG-2,3) DNA can be cut with restriction enzymes is visible from at least smear results of electrophoresis bands after DNA cut with *Eco*RI enzyme . *Eco*RI produce DNA bands when smears were electrophoreses because this restriction enzyme included in the frequent cutter. Ladder marker used 9,824bp. (FIG-4) The viral genome was not amplified using specific primers.771 bp ladder marker used. (FIG-5) No amplification was found after using DNA-A specific primers. The Phenol-based method facilitated quick and effective removal of cellular debris from the slurry as compared to Potassium acetate. But the amount of DNA precipitated was very low. Unavailability of any salt to help in precipitation was considered to be one of the main reasons for such an observation. The quality of DNA obtained from this method was satisfactory. The quantity (concentration) and quality of DNA determined by UV-Vis spectrophotometer at wavelength 260 and 280 nm. Determination of the total DNA quantity was calculated based on the value of absorbance at a wavelength of 260 nm. The highest DNA purity can be seen in the A260/280 ratio that produces the value of 1.8 to 2. Since the quality of DNA obtained from alcohol method was the best, so the quantification of all the samples of DNA samples obtained and quantification was done using nanodrop Spectrophotometer. Also these samples were only used for further study. The observations of nanodrop are as follows:(Table-1)

Jatropha have a high nutritional and economic value in India. With improved cultivars, especially hybrid cultivars, and higher input use and intensification of cultural practices, the yield level can be increased considerably. This is an exciting time for geminivirus research. Past work has taught us much about the mechanism of viral DNA replication and about the general roles of viral proteins in this process and has paved the way for new and important questions. What are the precise functions and activities of viral proteins involved in replication? How are these activities regulated in multifunctional viral proteins? How do viral proteins interact with host proteins during replication, and how do these interactions subvert the host replication machinery for the purpose of viral DNA replication.[9, 10] Geminivirus replication, as might be expected, appears to occur preferentially in cells that are actively synthesizing cellular DNA.

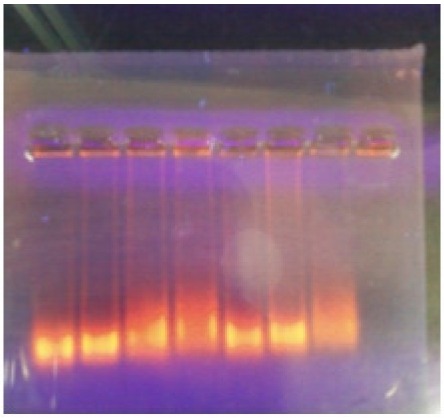
Recent evidence suggests that Rep can induce the accumulation of proliferating cell nuclear antigen (PCNA, a polymerase-6 processivity factor) in transgenic tobacco plants. Do geminiviruses, like the mammalian DNA tumor viruses, possess the ability to prepare the host for viral DNA replication by stimulating normally quiescent cells to enter S phase? If so, what other viral and host proteins are involved? These and other questions are currently under investigation in a number of laboratories. What is learned during the next few years promises to teach us a great deal more about the mechanisms of viral DNA replication and pathogenesis and will provide new insights into host-pathogen interactions and the fundamental mechanisms of plant DNA replication and its control. Also a new success in the study of geminivirus is obtained with the discovery of the phenomenon of RNA silencing [**11**] Begomoviruses are also, both inducers and targets of RNAi. The begomovirus siRNAs are of 21, 22 and 24 nucleotide in length. Moreover, many segments of the viral DNAs also are methylated in a siRNA dependent manner in response to infection. However, unlike the case in mammalian systems, the host microRNAs that interfere with replication and spread of plant viruses are not known yet.[12] In response to plant antiviral RNA silencing, viruses are not behind in waging an arms race to neutralize host defenses. They have evolved several RNAi evading mechanisms like evolution of siRNA resistant satellite genomes, defective interfering RNAs, loss of target sequences by high mutation rate, formation of RISC-inaccessible secondary structures, associating with protein complexes posing steric hindrance, encapsidation and partitioning their replicative cycles in vesicles, chloroplasts and nucleus. Suppressors can reverse gene silencing effects and allow high transgene expression – a desired goal of molecular farming. Thus, RNAi suppressors and their hosts with antiviral RNAi, the former seems to be having an edge, at least as seen from the human angle. The enormous loss of our crops to begomoviral diseases necessitates development of intervention strategies to efficiently contain the virus.

Spray of insecticides to get rid of the virus transmitting whitefly vector, is neither an effective nor an eco-friendly approach. Unfortunately, stable natural resistance sources for begomoviruses are few and plant breeders have not been successful in introgressing these largely multigenic traits into elite cultivars. Hence, modern biotechnology needs to offer an attractive alternative of engineering begomovirus resistance through transgenic route.[13]Pathogen-derived resistance (PDR) through the expression of various full length/truncated or defective viral proteins like Rep mutants of maize steak virus has been achieved.

Table.1 The quantification of all the samples of DNA samples obtained and quantification was done using Nanodrop Spectrophotometer. Also these samples were only used for further study. The observations of Nanodrop are as follows

Sample	Absorbance A-260	Absorbance A-280	260/230	260/280	Concentration Ng/µl
BKT-H	1.628	0.944	1.72	0.87	105.5
BKT-I	1.944	1.037	1.87	1.78	97.2
IT-H	1.558	0.811	1.92	0.92	112.7
IT-I	2.125	1.269	1.67	1.12	106.2
CH-H	2.14	1.623	1.80	1.34	98.0
CH-I	1.334	0.981	1.76	1.11	100.9
RT-H	1.230	0.666	1.85	1.10	61.5
RT-I	3.374	1.912	1.76	0.73	168.7

Fig.1 Detection Primers



LANE 1:-BKT HEALTHY
LANE 2:-IT HEALTHY
LANE 3:-CH HEALTHY
LANE 4:-BKT INFECTED
LANE 5:-IT INFECTED
LANE 6:-CH INFECTED
LANE 7:-RT INFECTED

Fig.2 Result of restriction digestion

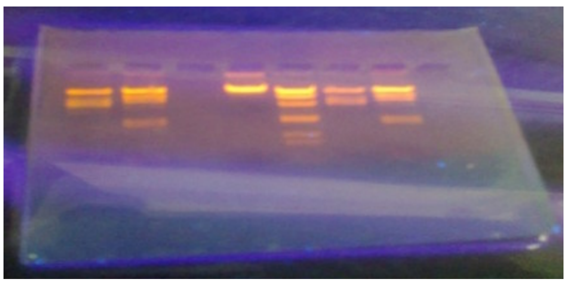


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Fig.3 Result of restriction digestion

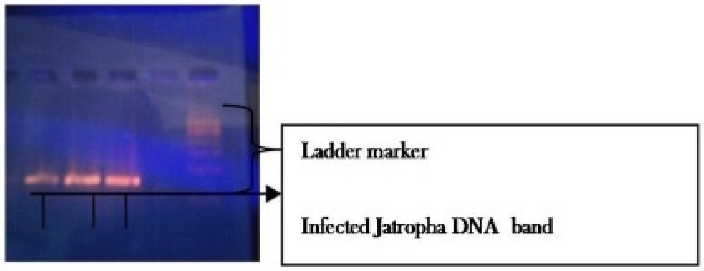


Fig.4 Result of PCR of infected jatropha plant

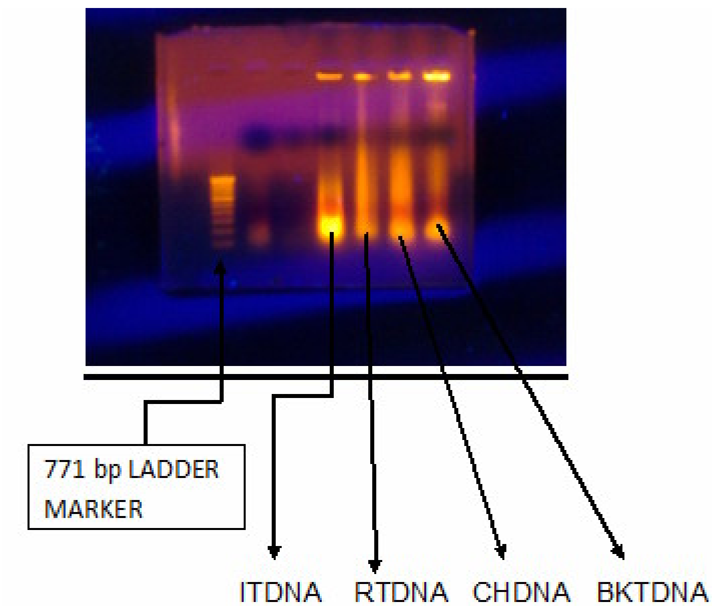
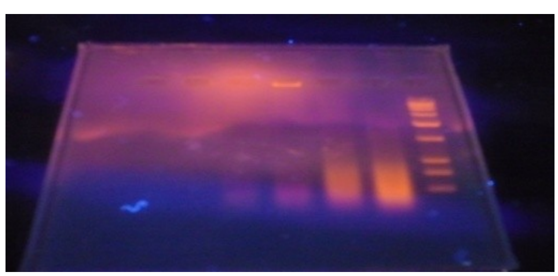


Fig.5 Result of rapd of infected jatropha sample DNA



Antisense and RNAi technology has also been used but without consistent success. An exciting new approach using another set of small RNAs called artificial microRNAs (amiRs) to achieve virus resistance has been proved successful for few viruses.[14] This approach has not been used on geminiviruses so far.

Apart from these strategies many other approaches like use of ribozymes, DNA binding proteins, peptide aptamers; GroEL, etc. have been attempted. A hammerhead ribozyme directed against Rep showed ~33% cleavage activity on synthetic *rep* transcript, while the *Bemisia tabaci* GroEL gene, expressed in transgenic tomatoes under the control of a phloem-specific promoter, protected the plants from yellow leaf curl infection. All these strategies have their share of advantages as well as disadvantages and it seems that stacking together of multiple approaches would only provide a durable resistance against begomoviruses, given their extremely high penchant for rapid mutation and recombination.

To conclude, our finding Diseases caused by geminiviruses have long been recognized as a limitation to the cultivation of several important crops, including maize, cassava; bean, squash, cucurbits, and tomato, in tropical and subtropical regions of the world. More recently, geminivirus diseases, particularly those transmitted by whiteflies, have become an even greater threat to agriculture due to the appearance of a new and more aggressive whitefly biotype. This has renewed interest in the study of geminivirus pathogenesis and epidemiology and has stimulated work on the development of virus-resistant crop plants. Due to all these factors, geminivirus was chosen as the subject of study.

During the tenure of this training, following achievements were credited, Recognition of disease symptoms in field samples was done. Total genomic DNA was isolated from the diseased samples. It was shown that the method employing Potassium acetate was most potent for the isolation of DNA under the present laboratory conditions and facilities provided. The viral genome was not amplified using specific primers. No amplification was found after using DNA-A specific primers

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