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RESEARCH ARTICLE

RECOMBINANT CROTON YELLOW VEIN MOSAIC VIRUS ASSOCIATED WITH SEVERE LEAF CURL DISEASE OF PAPAYA IN INDIA.

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Abstract

In this study, the infection of Begomovirus in Papaya (*Carica papaya*) plants from New Delhi was suspected in area exhibiting leaf curl disease. Rolling circle amplification and restriction fragment length polymorphism analysis suggested that a begomovirus was present in symptomatic plants. The full length sequence of a begomovirus DNA component was determined comprising 2745 nucleotides. Pairwise sequence comparison of the virus under study with other begomoviruses, performed using the species demarcation tool, suggested <92% pairwise nucleotide identity with Croton yellow vein mosaic virus (CYVMV). Notably, no other genomic components, including satellites, were obtained. Further nucleotide sequence comparison indicated its recombinant origin. Infectious clone of the begomovirus was constructed and inoculated into the leaves of *Nicotiana benthamiana* using a needleless syringe, and this resulted in a mosaic pattern and slight curling of the leaves.

Introduction:-

Papaya (*Carica papaya L.*), one of the most essential horticultural crop in India, is valued for the medicinal compounds present in various parts of the plant; these compounds are highly effective against diseases, such as dengue, cancer, malaria, and diabetes (Subenthiran et al., 2013; Melarriri et al., 2011; Yusha et al., 2009; Tarkang et al., 2013). Papaya, believed to be a native plant of Southern Mexico and Central America, is now grown worldwide including India. Papaya plants are in constant threat of several pathogens and among them a vector mediated virus, leaf curl virus is now a major concern. In India, leaf curl disease of papaya was first reported by Thomas and Krishnaswamy [1939]. The adverse effect of this disease can be easily observed in papaya growing fields, where most papaya plants are destroyed by this disease (Singh et al., 2008). Furthermore, several reports have suggested that this disease is also widely distributed in other countries, including Pakistan, China, and those in Africa (Nadeem et al., 1997; Zhang et al., 2005; Taylor et al., 2001). Plant viruses are divided into more than 15 families, of which Geminiviridae constitutes the largest single-stranded DNA (ssDNA) plant virus family (Yadava et al., 2010). Geminiviruses are characterized by their circular ssDNA genomes encapsidated in twinned quasi-isometric particles and whitefly (*Bemisia tabaci*)-mediated transmissibility (Mansoor et al., 2003; Dijkstra and khan, 2006). The family is divided into seven genera Mastrevirus, Begomovirus, Curtovirus, Topocuvirus, Becurtovirus, Eragrovirus, and Turncurtovirus on the basis of their genome organization, insect vector, host ranges, and genome arrangement (Varsani et al., 2014). Begomovirus is the largest genus among Geminiviridae, which is responsible for considerable agroeconomic losses worldwide (Brown et al., 2001). In plants, the symptoms that develop following begomovirus infection include downward and upward leaf curling, twisted petioles, venation, and stunting. On the basis of the typical leaf curling symptoms caused by this genus, the resulting disease is termed leaf curl disease. Leaf curl disease of papaya is a begomoviral disease which produces small and distorted fruits that tend to fall prematurely. Because begomoviruses have a high tendency of recombination, they have a wide host range. Papaya

plant is infected by several established geminiviruses species such as Cotton leaf curl Multan virus, Tomato leaf curl New Delhi virus (ToLCNDV), and Chilli leaf curl virus (ChLCV) (Sinha et al., 2013; Murthi et al., 2007; Raj et al., 2008). In this report, we have described the infection of CYVMV which causes leaf curl disease of papaya. In India as well as in many other countries, several recombinant viruses from the Geminiviridae family have been reported, particularly Cotton leaf curl virus, Tomato leaf curl virus (ToLCV), and Cassava mosaic virus (Singh- Pant et al., 2012).

Materials and methods:-

Sample collection:-

Four papaya leaf samples having typical leaf curl symptoms were collected from different papaya plants from two fields in New Delhi and stored at −80°C until required. One leaf from normal papaya plant was also taken as control.

DNA isolation and PCR:-

For formulating a comprehensive and effective strategy to combat this disease, the molecular characterization of the genomic components associated with this disease and their associated components is essential. Infected papaya leaves were collected from suburban New Delhi, India, during December 2012 (Fig. 1). Total nucleic acids were extracted from the leaves by the method of Dellaporta et al. (1983). Total DNA was subjected to rolling circle amplification (RCA) (Illustra TempliPhi Kit, GE Healthcare Life sciences, Buckinghamshire, UK).

Restriction digestion and cloning:-

The RCA product was digested into a single 2.7-kb fragment using *Sac*I. Same stretch of 2.7 kb was also found with other restriction enzyme *Xho*I but no restriction fragment was obtained with *Bam*HI. The obtained stretch with restriction enzyme *Sac*I was ligated into the respective site of vector pGreen0029 following standard procedure.

Bioinformatic analysis:-

The molecular characterization of the full-length DNA sequence was performed using the following bioinformatics tools: ORF finder tool, Blastn, Clustal W, and ExPASy protein server tool. For alignment and pairwise comparison, the species demarcation tool (SDT) was used (Kumar et al., 2011; Muhire et al., 2013). The evolutionary history of the virus under study was determined by analyzing its DNA, RNA, and protein sequences using MEGA 4.0 version software. The probability of recombination was determined using a recombination detection program (RDP 4.4.3, http://darwin.uvigo.es/rdp/rdp.html).

Amplification of satellite molecules:-

Furthermore, we used the DNA-B-specific primer PCRc1/PBL1v2040, betasatellite and alphasatellite DNA-specific primers for amplifying the DNA-B component, betasatellite and alphasatellite DNA, respectively (Rojas et al., 1993; Briddon et al., 2003; Bull et al., 2003). DNA-B, betasatellite and alphasatellite DNAs could not be amplified from any of the samples, indicating lack of association.

Preparation of agroinfectious clone:-

For preparation of agroinfectious clone, a primer pair was designed on the basis of sequence obtained above. Total DNA above isolated was used as the template and amplified using specific primers with *Sac*I/*Bam*HI restriction sites, FL/RFL: 5′-CGAGCTCCGCCCGCTTCGAAGGTACGTCGCC-3′/5′-CGGATCCGTGGAAATGATTATATCTGCTGG-3′. The PCR product was cloned in the pGEMT-easy vector, which was further digested using *Sac*I and *Bam*HI and ligated at the respective restriction sites of the pGREEN 0029 vector. This ligated vector was termed as pGREEN-IA. In the second step, the 2.7kb stretch (obtained from RCA digestion above) ligated in the *Sac*I site of pGreen0029 was digested with same enzyme (*Sac*I), and purified. Further, pGreen IA was digested with *Sac*I, and at this site purified stretch obtained from second step was ligated into the *Sac*I restriction site of the pGREEN-IA using T_4 DNA ligase resulting in the formation of a dimer, pGREEN-2A. The dimer was transformed into *Agrobacterium tumefaciens* GV3101, as described by Hofgen and Willmitzer (1988) and the helper plasmid pSoup was mobilized accordingly. On the other hand, pGREEN 0029 without the insert was separately transformed as a negative control.

Infectivity testing:-
Infectivity testing was performed by inoculating the agroinfectious clone into five *Nicotiana benthamiana* plants using a needleless syringe (Diego et al., 2006). Agroinfiltrated plants were maintained inside a glasshouse under standard conditions and monitored for 4 weeks for symptom development. One mock inoculated plant was grown simultaneously. Agroinoculated plants were screened using PCR and specific primers F1(for)/F1(rev) to determine viral DNA replication , and the PCR products were analyzed using 1% agarose gel electrophoresis.

Results and discussion:-

Nucleotide sequencing:-
The sequences obtained from all the clones (from different plant samples) were 99%–100% similar to each other. Therefore, the sequence of only one Begomovirus was submitted to NCBI GenBank under the name Croton Yellow Vein Mosaic Virus [Pap:ND:13] and accession number KF307208 was obtained for it. The viral genome (approximately 2.7 kb) consisted of seven ORFs: two in the virion sense direction and five in the complementary sense direction AV1 (coat protein), AV2 (precoat protein), AC1 (replicase), AC2 (transcription activator), AC3 (replication enhancer), AC4 (regulatory protein) and AC5.

Bioinformatic analysis:-
According to the BLASTn results, the virus under study shared 95%, 87%, 85%, and 86% nucleotide sequence identities with Catharanthus yellow vein mosaic virus (CaYMV), Papaya leaf curl virus (PaLCuV), Tomato leaf curl New Delhi virus (ToLCNDV) and Chilli leaf curl virus (ChLCV) respectively. Nucleotide sequence alignment and pairwise sequence comparisons were performed using the SDT. According to the results, the full-length genome of the virus under study (KF307208) showed <94% pairwise nucleotide sequence identities with other analyzed Begomovirus sequences. According to the guidelines of the Geminiviridae Study Group of the International Committee on Taxonomy of Viruses, the presently applicable strain demarcation threshold is 94% for Begomovirus (Brown et al., 2015). Thus, we concluded that the virus under study is a new Begomovirus strain of croton yellow vein mosaic virus which infects papaya plants in natural condition.

DNA-B and betasatellite DNA could not be amplified from any of the samples, indicating lack of association.

Phylogenetic analysis:-
Phylogenetic analysis demonstrated that this virus has evolved from Chili leaf curl virus, Catharanthus yellow mosaic virus, Pepper leaf curl virus, Ageratum enation virus, Tomato leaf curl virus, Papaya leaf curl virus, Radish leaf curl virus and other viruses. A phylogenetic tree was constructed using MEGA 4.0 software. According to the tree (Fig. 2), the virus under study was evolutionarily closest to CYVMV (JN817517), followed by ToLCuNDV (DQ629102) and PaLCuV (HM143914), all isolated from India.

Detection of recombination:-
Recombination majorly causes the emergence of new begomoviruses, which can infect a multiple host range. Assuming that this phenomenon is the cause of emergence of the virus under study, we performed analysis by using RDP. The results indicated that PaLCuNDV was recombinant, showing five recombination spots corresponding to Catharanthus yellow vein mosaic virus (CaYMV), Chilli leaf curl virus (ChLCV),Bhendi yellow vein Bhubaneswar virus (BYVBV), and Papaya leaf curl virus (PaLCV). The major parent was PaLCuV, whereas the minor was ChLCV. The details of recombination regions and parents, with P values are listed in Table 1.

Infectivity testing by agroinfectious clone:-
Infectivity testing by agroinoculating pGREEN-2A into *N. benthamiana* leaves showed that after 3 and half weeks, all five *N. benthamiana* plants started developing a mosaic pattern and slight curling, whereas the plants injected with mock clones showed no symptoms (Fig. 3a-3c). Furthermore, we confirmed the presence of Begomovirus in the tested leaf samples because the desired 1.2-kb amplicon was obtained through PCR using F1 (for)/F1(rev) primers (Fig. 3d). To check the virus replication, we have isolated total DNA from test plant and control plant and performed RCA. This RCA product was partially digested with *Sac*I, and stretch of 2.7 kb was again obtained from test plant (data not given). No result was obtained from control plant.

CYVMV is known to occur with yellow vein mosaic disease of weed *Croton bonplandianum* (Varma, 1963).In one of the reports by Pramesh et al., 2013. CYVMV was successfully transmitted by whitefly (*Bemisia tabaci*) on 35

plant species belongs to 11 families, including papaya also. Our finding demonstrated natural infection of CYVMV without betasatellite in papaya plants.

Across India, leaf curl disease of papaya continually destroys papaya plants in papaya growing fields. In this present study, we demonstrated that CYVMV infecting papaya is a monopartite virus from the Old World not possessing satellite DNA. The ability to induce symptoms in the naturally occurring *N. benthamiana* through an agroinfectious clone, even in the absence of a satellite molecule, indicates virulence capacity of this virus. To the best of our knowledge, it is the first report of naturally infecting CYVMV isolated from papaya.

Fig. 1 A: Figure of naturally occuring papaya plant.
Fig. 1B.: Begomovirus infected papaya leaf taken for this study showing typical leaf curl symptom.

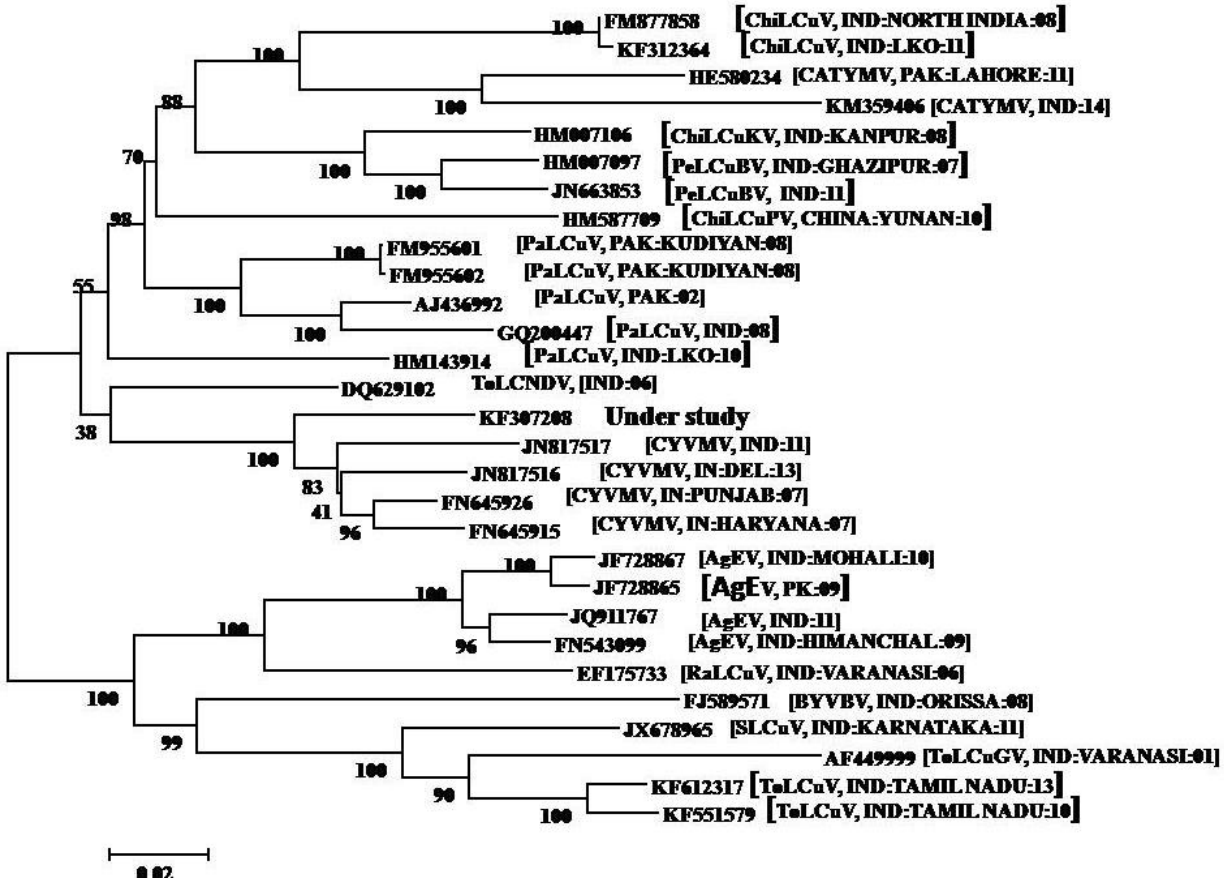

Fig. 2: Phylogenetic tree of papaya leaf curl New Delhi virus (KF307208) with other Begomoviruses from NCBI GenBank. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum

of branch length = 1.42565928 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method.

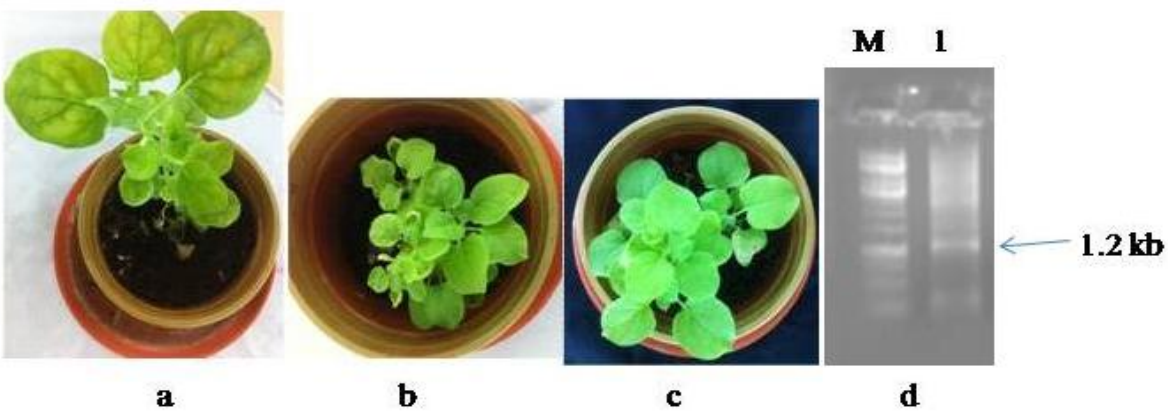

Fig. 3: Agroinoculation of dimer and empty vector in the leaves of N. benthamiana. The pictures were taken after three weeks post inoculation. Leaves show yellowing (A) and slight curling (B), while control plant inoculated with empty vector showed no symptom (C). Total DNA isolated from the agroinoculated leaves (test plant and control plant) was subjected to PCR with primer pair F1for/ F1rev, gave desired band of 1.2 kb (D, Lane 1) while DNA isolated from control plant gave no amplification (D, Lane 2). M: 1 kb Marker.

Table 1: Detection of recombination events using RDP 4.4.3 and details of nucleotide sequence detected as probable major and minor parents

Viral DNA segment	Nucleotide coordinate	Major parent- Acronym	Minor parent- Acronym	Average p value[a]
Full length genome of PaLCuV New Delhi isolate	36-1129	FM955601 PaLCuV	FJ589571 BYVBV	$5.849*1o^{-204}$
	601-1071	FM877858 ChLCuV	KF312364 ChLCuV	$6.698*10^{-62}$
	1072-1179	FM955602 PaLCuV	HM007106 ChLCuV	$8.181*10^{-06}$
	2184-2608	HE580234 CaYVMV	KF312364 ChLCuV	$1.151*10-^{05}$
	2720-2744	FM955601 PaLCuV	JN817517 CrYVMV	$1.890*10^{-20}$

[a] p values of the recombinant fragments

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