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Infectivity Analysis of Geminiviruses DNA Extract in Cotton and Tobacco

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ABSTRACT

The present study was planned to induce the disease symptoms in cotton and tobacco by inoculation of different combinations of inoculates e.g. partial dimers of cotton leaf curl virus (CLCuV), viral DNA, total genomic DNA, ccc DNA, plant sap under laboratory conditions. The infectivity of these components was determined by their capability to replicate upon introduction into cotton and tobacco. Samples from infected plants were collected from the fields. Genomic DNA of Geminivirus was extracted and replication study of inoculated virus was carried out by PCR technique. Clear replication of both Gemini virus variants and DNA-1 was detected both in cotton and tobacco but the mild symptoms appeared only in tobacco. This showed the possibility of involvement of other DNA component in leaf curl disease of cotton.

Key Words: Geminiviruses; DNA; Cotton; Tobacco

INTRODUCTION

Leaf curl disease of cotton is causing heavy losses to the cotton crop in Pakistan. It is estimated that during the last five years, the disease has resulted in a loss of 7.4 million bales of cotton with estimated value of US$ 4.98 billion. Recently, the disease has spread to Sindh which was previously free of disease (Mansoor *et al.*, 1998). The disease is associated with whitefly transmitted Geminivirus (Mansoor *et al.*, 1993; Hameed *et al*., 1994). It has been found that leaf curl disease of cotton is associated with variable Geminiviruses, but none yet have been unequivocally proved to be associated with infectivity (Nadeem, 1995; Harrison *et al.*, 1997; Zhou *et al*., 1997). During recent investigations, a novel circular viral DNA in naturally infected cotton plants was identified which show typical symptoms of leaf curl disease (Mansoor *et al.*, 1999). Now, it has been proposed that the nanovirus like component have a role in symptoms development. However, reinoculation of Geminivirus DNA and nanovirus like DNA-1 failed to induce wild type symptoms in cotton and tobacco.

Therefore, this study was initiated to search or narrow down the search for casual agent(s) related to the leaf curl disease. In this study, different isolations of Geminiviral DNA extracts have been used and reintroduced into the healthy cotton and tobacco plants though biolistic procedure.

MATERIALS AND METHODS

Sample collection. Fresh and young leaves from infected plants were collected in liquid nitrogen from the fields of Nuclear Institute of Biotechnology and Genetic Engineering (NIBGE).

Growing of cotton and tobacco seedling. The protocol used for the growth of seedling in jars was according to Murashige and Skoog (1962). Seeds were placed on Murashige and Skoog (MSO) medium. After almost one week, seeds were transferred into magenta jars for further growth into healthy plants.

Infected sources. Six infected sources total genomic DNA (Doyle & Doyle, 1990), viral particle (Czosnek *et al.*, 1988), viral DNA (Zeidan & Czosnek, 1991), ccc DNA (covolently closed circular) (Mansoor *et al*., 1999) whitefly DNA (Zeidan & Czosnck, 1991) and plant sap (Mathews, 1993) were extracted.

Biolistic inoculation. Seeds of cotton (NIAB-78) and tobacco (*N. benthemiana*) were germinated in MSO medium upto 3-4 leaf stage. These plants were bombarded through particle bombardment using PDS-1000/He from BioRad. Plants were inoculated with cccDNA, total genomic DNA, virus particle, viral DNA, whitefly DNA and plant sap. These were bombarded individually or in different combinations.

PCR based identification of CLCu disease related components. To confirm the presence and replication of viral DNA, total gnomic DNA was isolated from newly grown leaves of bombarded plants (Doyle & Doyle, 1990). Multiplex PCR was used to simultaneously detect the two viral species in a single reaction for a given DNA sample. Two oligo-nucleotide primers CLCR5′-AACTCGTAACCTAGGGTATCCGGTGAA3′ and VBTB 5′-CTTGAGCTCCAGTGATGGGTTCCCCTGTG 3′ were designed specific to each virus species and a third oligo-nucleotide primer PCL2 5′-CATGCCTCAAAA GCGGAACGGTATTTATT3′ common to both species. The species specific oligo-nucleotide primers were designated in conserved region of two CLCuV species and were sufficiently spaced to give different sized fragments for each species by PCR amplification. The primer CLCR and VBTB amplifies CLCuV-PK1/FSD/2 and primer PCL2 and VBTB amplifies CLCuV-PK2/FSD/I; whereas, primer VBTB is common primer for two species.

Reagents and profile used in PCR were 5µL DNA template, 5µL dNTPs (2.5 mM), 5µL of 10X PCR buffer, 3µL $MgCl_2$ (25 mM), 6µL of 3 multiplex primers, 0.5 µL Taq polymerase, 25.5 µL dH_2O. Total volume of PCR mixture was 50 µL. The profile used was initial denaturation temperature 94°C for 5 min, denaturation temperature 94°C for 1 min, anneal temperature 48°C for 1 min, extension temperature 72°C for 1 min and final extension temperature for 10 min.

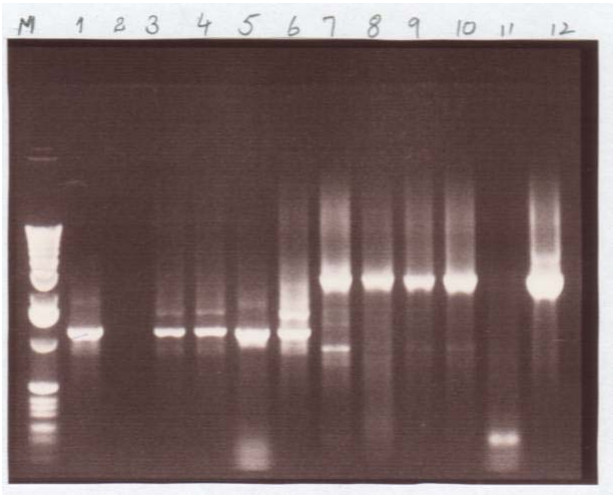
Primers for nanovirus like DNA-1 were PROI F (5′AAGGTACCAAAAAAACAATCTCCCTCATAC 3′) in the virion sense and PROI R (5′-TCAGATCTACCATCTTCGATTIAAGAACACAG 3′) in the complementary sense. PCR amplification was performed for DNA-1 using the primers DNA 1-F and DNA 1-R. The profile and reagents except primers were same.

Gel electrophoresis. After completion of PCR, 25µL of samples were analyzed by 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

All the DNA extracts clearly indicated the two strains of CLCuV and full length amplification (Fig. 1) as well as amplification of nanovirus DNA-1 like components (Fig. 2).

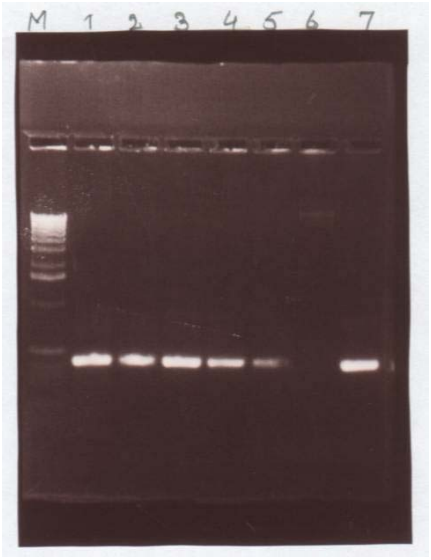
Fig. 1. Amplification products of PCR of all DNA extracts using full length and species specific primers for the differentiation of two CLCuV species and full length detection



Lane M= 1 Kb marker; Lane 1, 3, 4, 5= total genomic, ccc DNA, whitefly DNA, virus DNA, respectively indicated two species of CLCuV at 1.2Kb and 1.5Kb; Lane 6= partial dimmers; Lane 7, 8, 9, 10= full length detection of CLCuV in total genomic DNA, ccc DNA, whitefly DNA, virus DNA, respectively; Lane 2 and 11= negative control; Lane 12= positive control

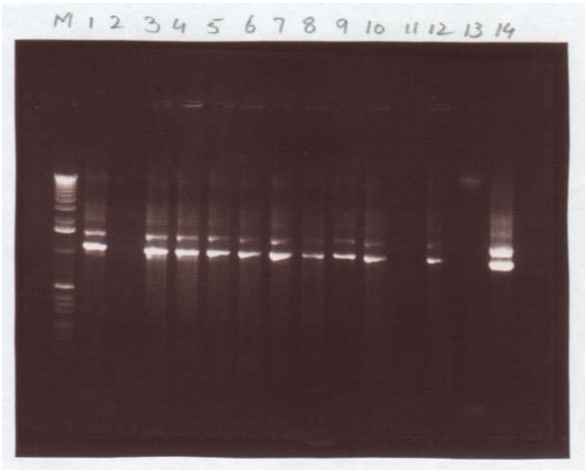
The results have been presented in Table I and II. The amplification products of multiplex PCR were 1.2 Kb and 1.5 Kb, respectively (Fig. 3).

Fig. 2. Amplification products of PCR from different DNA extracts using DNA 1 specific primers



Lane M= 1Kb DNA marker; Lane 1= total genomic DNA; Lane 2-3= ccc DNA; Lane 4= virus DNA; Lane 5= whitefly DNA; Lane 6= negative control; Lane 7= positive control DNA 1 clone.

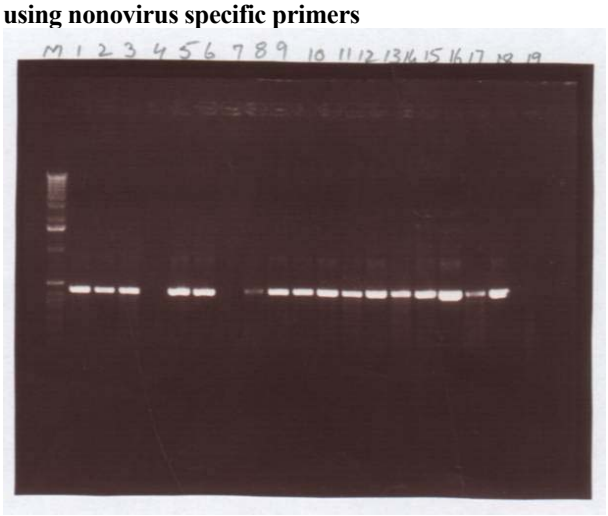
Fig. 3. Amplification products of multiplex PCR using common and species specific primers in cotton and tobacco plants bombarded by both different infected sources



Lane M= 1Kb marker 1; Lane 1-6= cotton plants bombarded by whitefly, total genomic, viral DNA, ccc DNA, plant sap partial dimmer, respectively; Lane 7-12= tobacco plants bombarded by whitefly DNA, total genomic, ccc DNA, viral DNA, partial dimer, plant sap respectively; Lane 13= negative control; Lane 14= positive control.

Similarly, PCR for nanovirus like DNA-1 also demonstrated their replication. Their amplification product was 0.5Kb in length (Fig. 4).

Fig. 4. Amplification products of different DNA extracts from inoculated cotton and tobacco plants using nonovirus specific primers



Lane M= 1Kb marker; Lane 1-2= ccc DNA; lane 3-4= total genomic DNA; Lane 5 to 8= plant sap, whitefly DNA, plant sap, viral DNA, respectively in cotton plants; Lane 9-11= plant sap, whitefly DNA, viral DNA, respectively; Lane 12-14= total genomic DNA; Lane 15-17= ccc DNA plants (in tobacco plants); Lane 18= positive control; Lane 19= negative control.

Very rich preparation of CLCu germinate particle from young field infected plant was obtained. Full length PCR amplification of 2.8Kb was obtained from all these extractions. As Liu *et al.* (1998) reported that after three months of infections, it is very difficult to amplify full length molecules of CLCuV. So full length amplification of Geminivirus from all the inoculum is very important as only full length DNA molecule can replicate in plants.

To study viral replication and movement in the inoculated plants two approaches can be used i.e. PCR and southern hybridization. Due to non-availability of radioactive labelled αP32 dATP only PCR analysis could be carried out. Symptoms development could not be observed in any of inoculated cotton plant even after two months of bombardment. However, in most of these plants Geminivirus like DNA-A and nanovirus like DNA-1 specific amplifications were observed in the newly growing leaves. Therefore, it may be concluded that CLCu Geminivirus DNA-A and nanovirus like DNA-1 alone cannot induce symptoms in cotton plants under lab conditions. In tobacco wild type symptoms were observed within one month in plants bombarded by ccc DNA. It has been suggested that bombardment of more number of plants with CLCuV ccc DNA extracts may induce symptoms in cotton. As CLCuV is complex of monopartite Geminivirus

Table I. Biolistic inoculation of different DNA extracts bombarded on cotton plants

Treat. No.	Inoculum/DNA extracts	DNA used per shot (conc.)	Cotton plants bombarded (No)	Replication/symptoms development in cotton
1.	Total genomic DNA	1.0µg	30	Replication of both CLCuV DNA-A as well as nanovirus DNA-1 like component was detected
2.	ccc DNA	1.0µg	30	"
3.	Virus particle	1.0µg	20	"
4.	Virus DNA	1.0µg	30	"
5.	Whitefly DNA	1.0µg	15	"
6.	Total genomic DNA bombarded + plant sap injected	0.8µg + sap	10	"
7.	ccc DNA bombarded + plant sap injected	0.8µg + sap	10	"
8.	CLCuV-Pak-2/FSD/1 CLCuV-Pak-1/FSD/2	0.5µg + 0.5µg	20	Replication of both CLCuV strains was observed
9.	CLCuV-Pak-2/FSD/1+ CLCuV-Pak-1/FSD/2 (bombarded) + plant sap (injected)	0.4µg + 0.4µg + sap	10	"

Table II. Biolistic inoculation of different DNA extracts from infeced cotton plants bombarded on + tobacco plants

Treat. No.	Inoculum/DNA extracts	DNA used per shot (conc.)	Tobacco plants bombarded (No)	Replication/symptoms development in tobacco plants
1.	ccc DNA	1.0µg	5	Replication and symptoms were observed
2.	Total genomic DNA	1.0µg	5	Replication of both CLCuV DNA-A as well as nanovirus like DNA-1 component was detected
3.	Virus particle	1.0µg	5	"
4.	Virus DNA	1.0µg	5	"
5.	Whitefly DNA	1.0µg	5	"
6.	Total genomic DNA bombarded + plant sap injected	0.8µg + sap	5	"
7.	ccc DNA bombarded + plant sap injected	0.8µg + sap	5	"
8.	CLCuV-Pak-2/FSD/1 CLCuV-Pak-1/FSD/2	0.5µg + 0.5µg	5	Replication of both CLCuV strains was detected
9.	CLCuV-Pak-2/FSD/1+ CLCuV-Pak-1/FSD/2 (bombarded) + plant sap injected	0.4µg + 0.4µg + sap	5	"

and nanovirus like DNA and so far neither infection clone of any nanovirus non-biolistic inoculation of nanovirus DNA extract is available. The role of protein component in symptom development has been established in some RNA viruses (Thornbury *et al.*, 1985) but this study was initiated with the hypothesis that DNA virus is involved in CLCuV disease, however, the possibility of RNA virus can not be ruled out until experimentally proved otherwise.

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