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Polymerase Chain Reaction-based Detection of Cotton Leaf Curl and Other Whitefly-transmitted Geminiviruses from Sindh

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Abstract

Samples of cotton plants showing symptoms of cotton leaf curl disease were collected from cotton fields in Sindh. Samples of some other plants including tomato, chillies, okra and *Hibiscus* suspected for whitefly-transmitted geminiviruses were also collected from these areas and total DNA was extracted. Degenerate primers designed to amplify DNA-A of whitefly-transmitted geminiviruses were used in PCR for the amplification of viral DNA. A product of expected (1.4 kb) was obtained from all these samples which confirmed the infection of whitefly-transmitted geminiviruses. PCR primers specific for the two whitefly-transmitted geminiviruses species namely CLCuV-Pk1 and CLCuV-Pk2 found associated with cotton leaf curl disease in Punjab were also used to confirm identity of cotton leaf curl virus in Sindh. A product specific for CLCuV-Pk1 was obtained from all four symptomatic cotton samples. The results showed that cotton samples were infected with CLCuV-Pk1 while CLCuV-Pk2 was not detected in these samples. This is the first report of detection of whitefly-transmitted geminiviruses on these crops from Sindh. Our data not only confirm the presence of a whitefly-transmitted geminivirus on cotton but also showed that the disease is caused by one of the virus species found in Punjab.

Key words: Leaf curl disease, geminiviruses, DNA, PCR, whitefly

Introduction

Cotton is the main cash crop of Pakistan which sustain major parts of country's economy. Sindh contributes about 20% of cotton production in Pakistan while rest is cultivated largely in Punjab. Cotton leaf curl disease has been causing heavy losses to cotton crop in Punjab (Mansoor *et al*., 1993a). It is estimated that in the last five years the disease has caused loss of 7.4 million bales of cotton with an estimated value of 4.98 billion US dollars. However, Sindh remained free of the disease. The symptoms of the disease were first observed in 1996 in Sindh in areas adjoining cotton-growing areas of Punjab. The symptoms are similar to the disease found in Punjab and include leaf curl, enations, vein thickening, and vein darkening. This year the disease has been observed in Sukkar and Khairpur districts on some plants. The symptoms similar to cotton leaf curl disease were also observed on *Hibiscus* in Ghotki, district Sukhar. It is suspected that the disease may acquire epidemic level in Sindh unless quick decision to stop disease spread are taken. Besides cotton there are other crops cultivated before cotton and are suspected for the presence of whitefly-transmitted geminiviruses. For the effective management of the disease, it is important to find out the causative agent. It was previously shown that cotton leaf curl disease in Pakistan is associated with a new whitefly-transmitted geminivirus (Mansoor *et al*., 1993b). Recently, two geminivirus species were identified which can cause the same disease independently but are often found to co-infect the same plant (Bashir *et al*., 1997).

Polymerase chain reaction (PCR) is a highly sensitive and reliable tool for the detection of plant viruses. Geminiviruses are small, single standard DNA viruses with circular genome and thus are well suited for the detection of these viruses by PCR. Several degenerate primers based on the conserved sequences of whitefly-transmitted geminiviruses have been designed for the detection of these viruses (Rojas *et al*., 1993; Briddon and Markham, 1994; Wyatt and Brown, 1996). These primers can amplify previously un-characterized geminiviruses. On the other hand PCR primers designed on the basis of non-conserved sequences can be used to specifically detect a particular virus or strain of the same virus (Rybicki and Hughes, 1990; McGovern *et al*., 1994).

In this communication samples showing possible geminiviruses infection were collected from Sindh and were confirmed for the presence of geminivirus by PCR using degenerate primers for whitefly-transmitted geminiviruses. Primers specific for two geminivirus species found in cotton growing areas of Punjab were used to confirm the identity of cotton leaf curl virus in Sindh.

Materials and Methods

Survey and sample collection

Survey of cotton growing areas of Sindh particularly areas closer to Punjab was conducted before cotton season and samples of plants suspected for the presence of whitefly-transmitted geminiviruses were collected. The name of plan species and symptom are given in Table 1. Young symptomatic cotton plants were collected from these areas during cotton season. Either whole plant or young leaves were brought to NIBGE in fresh condition.

Total DNA isolation

Total DNA was isolated from young symptomatic leaves (1-2 g) by modified CTAB methods as described previously (Doyle and Doyle, 1987). Briefly, leaves were ground to a fine powder in a pestle and mortar using liquid nitrogen. The tissue was mixed with hot DNA extraction buffer (60°C) and keep at 60°C for 30 minutes with shaking. The samples were extracted with chloroform isoamyl alcohol mixure (24:1). Total DNA was precipitated by using 2/3 volume isopropanol. DNA was pelleted by centrifugation and was dissolved in appropriate volume of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

PCR with degenerate primers for whitefly-transmitted geminiviruses

PCR primers designed on the basis of conserved sequences in replication associated protein (Rep protein) named WTGF (5'-GATTGTACGCGTCCDCCTTTAATTTGAAYBGG-3') and coat protein of whitefly-transmitted geminiviruses named WTGR (5'-TANACGCGTGGCTTCKRTACATGGGCCTDT-3') have been described previously (Mansoor *et al*., 1997b). PCR was carried out in a volume of 50 µL with buffer provided with the Taq polymerase (Amplitaq, USA). The final concentration of $MgCl_2$ ranged from 1.5 mM to 2.5 mM and greatly affected PCR amplification. PCR mix included 200 µM of dNTPs, 5 µM of each primer and 1.5 unit of Taq polymerase. PCR profile consisted of 1 min at 94°C, 1 min and 5°C and 2 min at 72°C for 40 cycles. In the last cycle the synthesis time was increased to 7 minutes and samples were cooled to 4°C unless removed from thermal cycler. PCR product was detected by agarose gel electrophoresis using Kb ladder as size standard.

PCR with primers specific for two cotton geminivirus species

A rapid multiplex PCR was used for the detection of two geminivirus species associated with cotton leaf curl disease. The design of PCR primers for multiplex PCR has been described elsewhere (Mansoor *et al*., 1997b). Briefly, a primer in the virus sense named CLCuV F (5'-GTGCTCAGATTTGCATTTAAATTATGAAATTG-3') was designed on the sequence common to two geminivirus species in C1 where reverse primer was designed on the sequences unique to two virus species. For CLCuV-Pk1 the primer was designed in the complementary sense at the start of C4 gene and is named as C4 Pk1 (5'-CGACCATGGGAGCCCTCATCTCCATGTGC-3') whereas the primer specific for CLCuV-Pk2 named PCL2 (5'-CATGCCTCCAAAGCGGAACGGTATTTATT-3') was designed at the start of C1 in complementary sense. The two products are distinguished on the basis of size difference on agarose gel. PCR reaction mixture contain 5 µM of three primers, 200 µM dNTPs, and final concentration of 2 mM of $MgCl_2$. Buffer provided with the Taq polymerase (Amplitaq, Perkin Elmer, USA) was used in the reaction. PCR was carried out in a thermal cycler with a profile of 30 sec at 94°C, 30 sec at 55°C, and 45 sec at 72°C for 40 cycles.

Results

Survey and symptomology of infected plants

The symptoms on cotton and other plants are shown in Fig. 1. Cotton samples were collected from four sites in the districts of Sukkar and Khairpur. The symptoms were recorded on a cotton varieties CIM 240, Shaheen and NIAB-78. The symptoms include leaf curling, vein thickening and enations. *Hibiscus* plant were collected with similar symptoms from Ghotki area. Tomato and chillies with leaf curl symptoms were collected from Nawabshah district. Okra with yellow vein symptoms was also collected from Nawabshah and Sukker districts.

Fig. 1: Symptoms of cotton leaf curl virus on cotton Sindh. Thick dark veins, leaf curling and enations are visible

PCR with degenerate primers for whitefly-transmitted geminiviruses

PCR with degenerate primers was carried out to confirm the infection of whitefly-transmitted geminiviruses on these samples. A product of expected size was obtained from all samples and confirmed the infection of geminivirus (Fig. 2). No amplification was obtained in healthy cotton plant whereas a product of expected size was obtained from CLCuV infected sample from Punjab which was used a positive control.

PCR with primers specific for two whitefly-transmitted geminivirus species

Multiplex PCR was used for the detection of two geminivirus species associated with cotton leaf curl disease in Punjab. A product specific for CLCuV-Pk1 was obtained from all four cotton samples whereas the product specific for CLCuV-Pk2 was not detected in these samples (Fig. 3). The same product was obtained from *Hibiscus* sample collected from Ghotki. Sample which was known to be infected with both geminivirus species was used as positive control and both viruses were detected by PCR (Fig. 4).

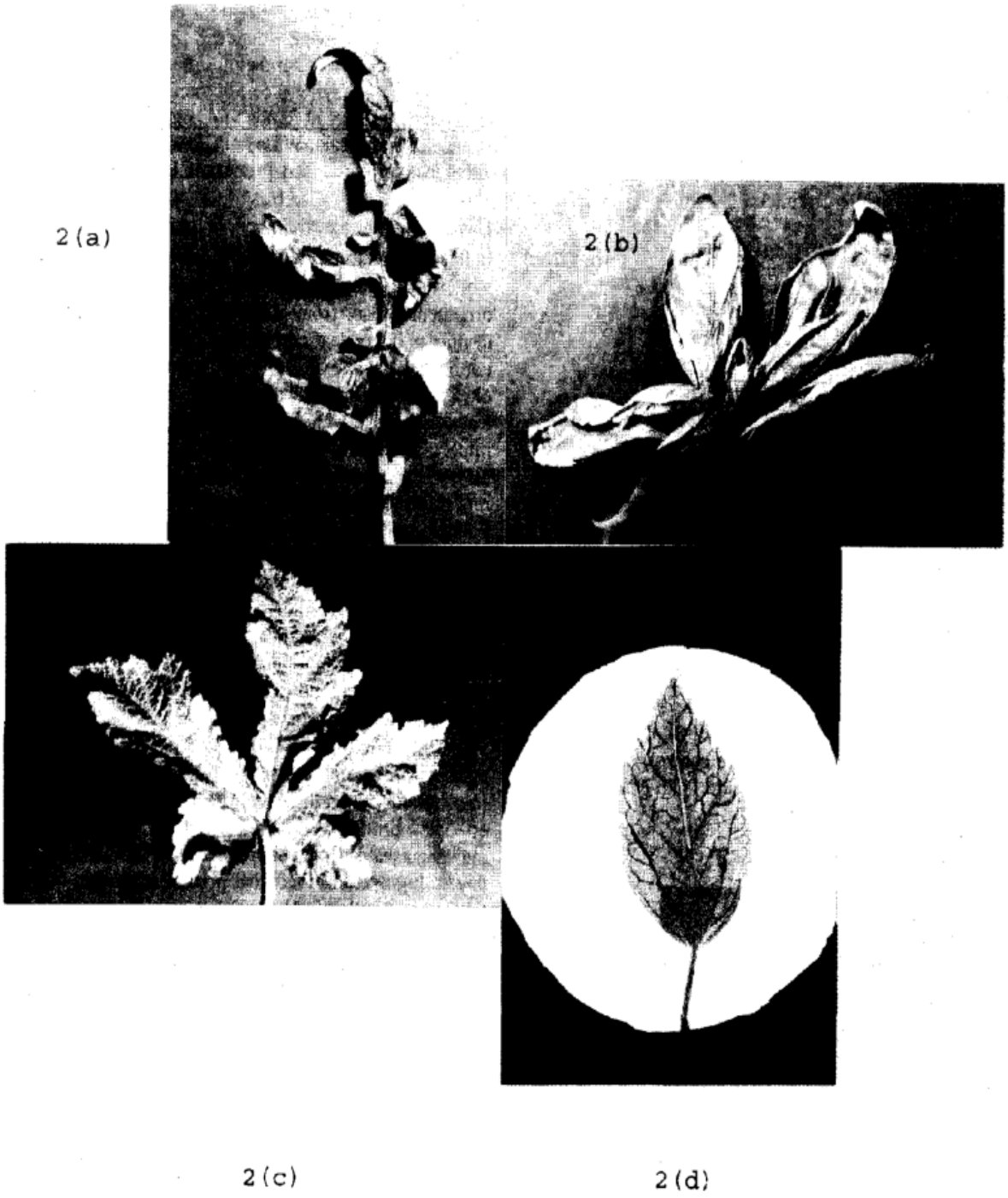

Fig. 2(a-d): Geminivirus infection on vegetable crops and ornamental plant from Sindh (a) Tomato showing leaf curl symptoms, (b) Chillies with leaf curl symptom, (c) Okra with yellow vein symptoms and (d) *Hibiscus* showing vein thickening and enations

Table 1: Plant species, symptoms and detection of whitefly-transmitted geminiviruses in plant samples from cotton-growing areas of Sindh

Plant species	Symptoms	Detection of virus by PCR		
		WTG	CLCuV-Pk1	CLCuV-Pk2
Cotton (*Gossypium hirsutum*)	LC, VT E	+	+	–
Cotton (*G. hirsutum*)	LC, VT E	+	+	–
Cotton (*G. hirsutum*)	LC, VT E	+	+	–
Cotton (*G. hirsutum*)	LC, VT E	+	+	–
China rose (*Hibiscus rosa-sinensis*)	LC, VT E	+	+	–
Tomato (*Lycopersicon esculentum*)	LC, VT	+	–	–
Chillies (*Capsicum annum*)	LC	+	–	–
Okra (*Hibiscus esculentus*)	YV, M	+	–	–

LC = Leaf curl, VT = Vein thickening, E = Enations, YV = Yellow vein, M = Mosaic, WTG = PCR with degenerate primers for whitefly-transmitted geminiviruses, CLCuV-Pk1 = PCR with cotton leaf curl virus Pk-1 primers, CLCuV-Pk2 = PCR with cotton leaf curl virus Pk-2 primers

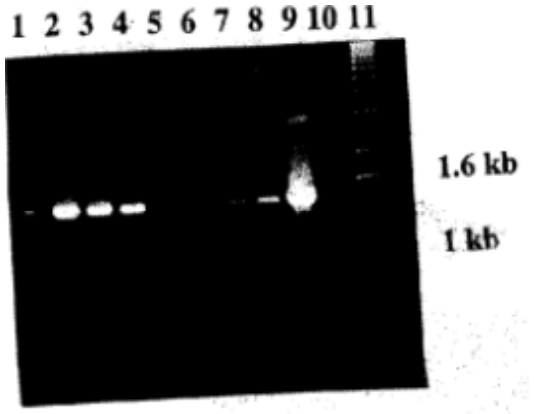

Fig. 3: PCR amplification of whitefly-transmitted geminivirus from Sindh Lane 1-4) cotton, 5) tomato, 6) chillies, 7) *Hibiscus*, 8) okra 9) infected cotton from Punjab (positive control), 10) Healthy cotton (negative control) and 11) kb DNA marker

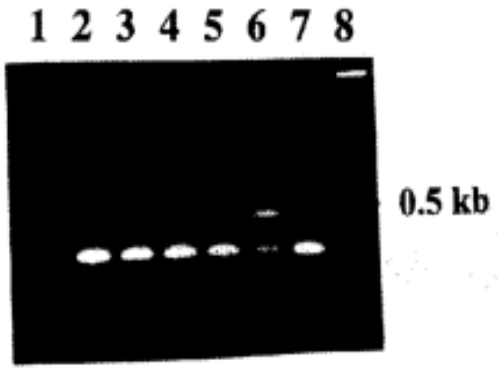

Fig. 4: PCR amplification for the detection of two cotton whitefly-transmitted geminiviruses from Sindh, Lane 1 healthy cotton, Lane 2-5 cotton samples, 6) cotton sample from Punjab infected with two virus, 7) *Hibiscus* and 8) kb marker

Discussion

In this communication we have used PCR for the detection of whitefly-transmitted geminiviruses on four crops and an ornamental plant from Sindh. We have also confirmed the identity of cotton leaf curl virus in Sindh and have shown that cotton is infected with CLCuV-Pk1. Tomato, chillies and okra are three important vegetable crops in Sindh. Tomato leaf curl virus, a whitefly-transmitted geminivirus in the most important constraint for tomato production in Sindh. The disease causes 30-40 percent yield losses. This is the first report of the detection of tomato leaf curl virus from Sindh. Earlier, we have shown that tomato leaf curl virus from Punjab is a bipartite geminivirus (Mansoor *et al*., 1997a). It will be interesting to known about the nature of tomato leaf curl virus found in Sindh. Chillies is another important in Sindh and leaf curl disease of chillies is a serious problem throughout Pakistan. Similarly okra is a widely cultivated vegetable crop in Sindh.

Okra yellow vein virus is the major virus infecting okra in Pakistan. Harrison *et al*. (1997) have recently determined epitope profile of cotton leaf curl and other whitefly-transmitted geminiviruses curl viruses from Pakistan. The epitope profile of okra yellow vein virus was indistinguishable from epitope profile of CLCuV and could be due to highly conserved coat protein or mixed infection of CLCuV and yellow vein virus. We have shown that probe of intergenic region of CLCuV did not hybridized with okra yellow vein virus (unpublished data) and the virus was not amplified by specific primers suggesting that okra yellow vein virus and CLCuV are two distinct viruses.

Cotton is an important cash crop in Sindh. In the last five years the production and share of Sindh in total cotton produced in Pakistan has increased significantly. This is attributed mainly to the fact that Sindh remained free of cotton leaf curl disease. We were interested to know the identity of the virus in Sindh. Nateshan *et al*. (1996) have shown that cotton leaf curl virus in Southern India is different from cotton leaf curl virus from Punjab. Similarly, there are two geminivirus species infecting cotton in Punjab, Pakistan. Our data shows that cotton leaf curl virus in Sindh is associated with CLCuVPk-1. This result is not surprising

as the disease was first observed in areas close to Punjab and suggest disease spread form Punjab. The appearance of cotton leaf curl disease in Sindh is a serious issue and require immediate work on etiology, epidemiology ad breeding for virus resistance.

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