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Full Length Article

Molecular Screening of Cotton Germplasm for Cotton Leaf Curl Disease Caused by Viral Strains

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Abstract

Cotton crop is infected by single stranded DNA viruses (Family, *Geminiviridae*) in Pakistan. Due to changing global climate, which favors the whitefly (insect vector) growth and reproduction, the spread of cotton leaf curl disease is increasing at an alarming rate. Various approaches are being used to diagnose the ssDNA viruses in cotton. One step PCR protocol was established to identify the three viruses, namely cotton leaf curl Multan virus (CLCuMuV), Burewala strain of Kokhran virus (CLCuKo-Bur) and cotton leaf curl Kokhran virus (CLCuKoV). Thirty eight genotypes were screened on the basis of symptoms and molecular diagnosis. Out of 38 genotypes, 13 were positive for CLCuMuV, 24 were positive for Burewala strain and one was positive for both CLCuMuV and CLCuKoV-Bur strain. However, none of the samples was positives for cotton leaf curl Kokhran virus. Our experiments have established one step guide PCR for screening of cotton germplasm for major cotton infecting viruses. This diagnostic assay will promote the breeding programs for the development of resistant cotton varieties against cotton leaf curl disease (CLCuD) in Pakistan. © 2017 Friends Science Publishers

Keywords: Begomovirus; Diagnostic primers; CLCuD; Disease index; One step guide PCR

Introduction

Cotton leaf curl disease (CLCuD) was first recorded in Nigeria in 1912 (Farquharson, 1912). Later, it was reported from Tanzania and Sudan (Jones and Mason, 1926; Bailey, 1934; Nour and Nour, 1964). CLCuD was first reported in Pakistan in 1967 in the vicinity of Multan in Tiba Sultan Pura as a minor disease (Hussain and Ali, 1975). Due to significant losses, it was considered as a major limiting factor for cotton production (Briddon and Markham, 2000). The disease started spreading during 1989 and appeared as epidemic in 1992–1993 and subsequent years. Consequently cotton production in Pakistan dropped from 12.8 million bales (1991–1992) to 9.05 million bales in 1992–1993 and 8.04 million bales in 1993–1994 (Annonymous, 2001). The epidemic of CLCuD during 1992 was found to be due to cotton leaf curl Multan virus (CLCuMuV) and the farmers faced losses of about 4.98 million bales amounting to US$5 billion from 1992–1997 (Briddon and Markham, 2000; Tahir *et al.*, 2011). After 1997, CLCuD spread in the Sindh Province (Pakistan), which was earlier free from this disease (Mansoor *et al.*, 1998).

Before the epidemic of 1993, the cultivars of cotton grown in Punjab served as silent or latent disease inoculum. For example, a variety S-12 was released in 1988 and grown on almost 46% area of the Punjab. The widespread adoption of this variety, that was highly susceptible to CLCuD, was considered as the source of epidemic. Some other widely grown varieties namely, CIM-70, NIAB-78, and CIM 240 were also susceptible to CLCuD (Iqbal *et al.*, 1997). Later in 1996–1997 some varieties were bred such as CIM-1100, CIM-448, CIM-446, CIM-443 and VH 53 showed tolerance to the disease (Iqbal *et al.*, 2014). Besides screening of the existing cultivars, efforts were made by cotton breeders for the identification of resistance sources from exotic germplasm. Furthermore, it was discovered that CP-15/2 and LRA-5166 (imported from India) possessed resistance against CLCuD, which was used in breeding programs for the development of resistant varieties (Rahman *et al.*, 2005). A CLCuD resistant variety "FH-634" was released in 1996 for general cultivation after the introduction of resistance gene from Cedix (Rashida *et al.*, 2005). In 1998, another variety "FH-53" was released with improved tolerance having resistant genes from Kivi-1021. During 2000 the varieties MNH-552, MNH- 554 and FH-900 with considerable resistance derived from LRA 5166 were released. Varieties namely FH-901 and BH-118 were also released for general cultivation having resistant genes from

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CP15/2 and Cedix respectively (Rashida *et al.*, 2005). Due to consistent efforts of breeders, cotton production was restored after the introduction of high yielding and CLCuD resistant varieties (Mansoor *et al.*, 2003). However, resistance breakdown occurred and all the resistant cotton varieties collapsed during 2001 because of the emergence of a new recombinant strain named Burewala strain (named after the Burewala area from where it was identified for the first time). After sequence comparison with other begomoviruses available in NCBI GenBank it was classified as a new strain of cotton leaf curl Kokhran virus i.e., CLCuKoV-Bur (Amrao *et al.*, 2010). Due to the epidemic of Burewala strain, all the resistance varieties became susceptible again (Iqbal *et al.*, 2014).

For screening, usually a highly susceptible cotton variety S-12 is used as spreader line and white fly as a vector for transmitting virus (Shah *et al.*, 2004; Rashida *et al.*, 2005). Another popular method for screening is to alter the sowing time of tested varieties i.e. early, normal and late sowing. In this procedure, a disease nursery is established adjacent to the experimental plot to allow the frequent movement of whitefly in the cotton fields during the season.

It is considered that early sowing is beneficial for cotton due to heavy attack of virus on late sown genotypes (Ahuja *et al.*, 2007; Perveen *et al.*, 2010). Grafting is accepted as an efficient method for screening against CLCuD. Virus is inoculated in test rootstock plants via infected scion and the response of genotypes can be observed within 14 days. Three types of grafting are generally used i.e. top cleft, bottle graft and wedge graft by using root stock as the potential resistant source and scion as disease inoculum (Shah *et al.*, 2004). All these methods end up with the visual observation of symptom development and rating the intensity of disease incidence phenotypically.

Phenotypic characterization of CLCuD has many limitations regarding accuracy and false negatives. This may lead to the selection of susceptible host plants as parents in a breeding program. Single host plants can harbor a number of viruses and this complicates the accuracy of disease diagnosis and properly correlating host plant genetics to disease resistance responses. Thus classification of varietal tolerance benefits greatly with virus confirmation by performing serological techniques like ELISA and/or PCR based techniques targeting the viral genomes (Mansoor *et al.*, 2003; Akhtar *et al.*, 2004, 2010; Shah *et al.*, 2004). The resistant varieties developed by employing these screening methods coupled with gene pyramiding, like MNH 886 and IUB 222, (Iqbal *et al.*, 2014) have contributed a lot in recovering cotton production and to combat the losses. However, cotton crop is still at a risk due to the presence of multiple begomoviruses in the field and the lack of using any standard molecular screening method to select the best resistant parent.

Due to small, circular single stranded nature of Geminiviruses, different viruses can be easily amplified by PCR by using degenerate primers (Rojas *et al.*, 1993; Briddon and Markham, 1994; Wyatt and Brown, 1996). But this procedure can be employed efficiently only to identify a class of viruses but a particular virus species or strain cannot be detected. In other words, particular causative agent is not identified until we clone and sequence that organism. One objective of this experiment was the evaluation of the current genetic diversity of viruses, implicated in CLCuD, among cotton germplasms in Pakistan. Another purpose was the development of a molecular screening method by designing viral strain specific primers in a single reaction of PCR. The endpoint objective was the development of one-step diagnostic tool for the rapid identification of diverse types of viruses, which will help to devise breeding strategy aiming at broad spectrum resistance.

Materials and Methods

Phenotypic Assessment of Cotton Germplasm for CLCuD

The present study was conducted on 38 genotypes of cotton (*Gossypium hirsutum* L.) germplasm maintained in the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad (Pakistan). All conventional agronomic and plant protection practices were followed to keep the genotypes in good condition. However, no pesticides were sprayed against whitefly to allow maximum population. The response of genotypes was monitored by using a disease scaling system (0–6) proposed by Akhtar *et al.*(2004) (Table 1).

Molecular Screening using Diagnostic Primers of CLCuMuV, CLCuKoV-Bur and CLCuKoV

Fresh leaves of cotton genotypes showing typical symptoms of CLCuD like curling, vein darkening, vein thickening and abnormal foliar growth, were collected from all genotypes and placed in Eppendorf tubes, labeled properly according to area, name, and date of collection, and then stored immediately in liquid nitrogen to avoid tissue damage. The samples were brought to lab for extraction of genomic DNA. Total DNA was extracted by using CTAB method (Doyle and Doyle, 1990).

Diagnostic primers, Beg-01 (3′ACGCGTGCCGTGCTGCTGCCCCCA5′) and Beg-02 (5′ACGCGTATGGGCTGYCGAAGTTSAGACG3′) were initially used for confirmation of begomoviruses in the genotypes (Briddon and Markham, 1994). Subsequently diagnostic primers were designed for the identification of specific species of begomoviruses associated with CLCuD. The sequence of the primers and the genes on which these primers are designed specifically for each of the CLCuKoV-Bur, CLCuMuV and CLCuKoV-Ko are presented in Table 2. Burewala strain is a recombinant strain and acquired the

Table 1: Scale for cotton leaf curl disease along with symptoms

Symptoms	Disease rating	Disease reaction
Complete absence of symptoms.	0	Immune
Thickening of a few small scattered veins	1	Highly resistant
Thickening of a small group of veins	2	Resistant
Thickening of all veins	3	Moderately resistant
Severe vein thickening and leaf curling at the top of the plant	4	Moderately susceptible
Severe vein thickening and leaf curling on the half of the plant	5	Susceptible
Severe vein thickening, leaf curling, and stunting of the plant with reduced fruit production.	6	Highly susceptible

Table 2: Specific primers used for amplification of specific species and strain of begomoviruses in cotton

Name of Primers	Primer Sequence	Primer range	Gene
CLCuKoV-Bur-F	5′GCAGTTAGTAGAGAAAACTTACTCTCC3′	206-232	V2
CLCuKoV-Bur-R	3′TATAGTAATTGAGGGAGATAGTCGAAC5′	1922-1948	C1
CLCuMuV-F	5′ CAACTTTTATCGCAGGATTATTCACC 3′	191-216	V2
CLCuKoV-R	3′ TCCTAATGAACAGAGACATCATCAC 5′	1324-1348	Ren and TrAP

virion strand from CLCuKoV-Ko and complimentary strand from CLCuMuV (Amrao *et al.*, 2010). Forward Primer (CLCuKoV-Bur-F) to amplify Burewala strain was designed on V2/Coat gene region and the sequence of this gene is acquired from CLCuKoV. Reverse primer (CLCuKoV-Bur-R) was designed on C1/Rep gene region because Burewala strain has acquired this region from CLCuMuV (Table 2). Forward primer (CLCuMuV-F) to amplify CLCuMuV was designed on V2 gene region which is specific for CLCuMuV and reverse primer (CLCuKoV-Bur-R). It was the same primer that we have used to amplify Burewala strain, (it was the C1/Rep sequence of CLCuMuV). Reverse primer (CLCuKoV-R) to amplify CLCuKoV is designed on REn+TrAP genes sequences which are specific to CLCuKoV. Forward primer (CLCuKoV-Bur-F) remained the same that was used to amplify Burewala strain.

The concentration of DNA in each sample was determined by spectrophotometer (Smart Spec TM Plus, BioRad) at 260 nm with conversion factor of OD of 1 = 50 µg/mL and 15–20 ng concentration of DNA was used for further experiments. The final single reaction mixture was prepared in 25 µL mixture containing Taq polymerase and its buffer, dNTPs and primers (Dream Taq, as recommended by Thermoscientific). The PCR profile was set at initial cycle for denaturation of DNA duplex at 94°C for 3 min followed by 25 cycles, each consisting of 1 min for denaturation of DNA at 94°C, 1 min for annealing of primers at 50°C, and 1 min for elongation of newly synthesizing DNA strand at 72°C, and final cycle of extension of DNA for 10 min at 72°C. The thermal cycler was set to hold at 4°C after completion of all cycles. Amplified product of PCR (2 µL) was mixed with 5X loading dye and electrophoresed in 1% (w/v) agarose gels containing ethidium bromide (0.5 µg/mL). Agarose Gel was viewed using a short wavelength ultraviolet (UV) trans-illuminator (Bio Red) and fragment length estimated by comparison with a co-electrophoresed 1kb DNA ladder (Fermentas).

Results

Screening of Germplasm against CLCuD Resistance

In the past decades, several cotton genotypes were successfully introduced in Pakistan for general cultivation by using conventional breeding approaches. Using the standard agronomic practices, the genotypes were grown at the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad. Genotypes were examined carefully to observe the disease incidence by using scale mentioned in Table 1. Although, all of the cotton genotypes (38) showed the typical symptoms of CLCuD, there was differential response against the disease nonetheless (Fig. 1). Some genotypes exhibited mild symptoms and some showed severe symptoms indicating that the response was tolerant to highly susceptible against CLCuD (Table 3). Out of 38, 12 genotypes showed mild symptoms and their disease rating scale was 1. Three varieties namely, PB-38, PB-39 and PB-896 developed by Department of Plant Breeding and Genetics showed better response against disease and the severity index was 1, 1 and 2, respectively. Cotton varieties, MNH-886 and FH-142 were ranked at disease severity scale 2, and these varieties are currently widely grown in fields. Only one variety (IR-6) showed the disease severity scale of 5.

Amplification of Begomoviruses

Initially all the samples of cotton were subjected to PCR amplification by using universal primers (begomo01 and begomo02) of begomoviruses (Briddon and Markham, 1994). All the infected plants showed amplification ~2.8kb size of begomoviruses. The amplified PCR product was run on gel along with +ve control (Fig. 2B, lanes 1 and 2). DNA marker of 1kb was also loaded on gel with PCR samples to reconfirm the 2.8 kb size of begomoviruses. This experiment confirmed that all the genotypes were infected by begomoviruses.

Table 3: Response of cotton germplasm and amplification of viruses associated with CLCuD

Genotypes	Disease scale	CLCuMuV	CLCuKoV-Bur	CLCuKoV
PB-38	1	-	+	-
PB-39	1	-	+	-
PB-896	2	-	+	-
AS-01	4	+	-	-
CIM-598	2	-	+	-
Coker 100/A2	2	-	+	-
La Frego3159	4	+	-	-
IR-6	5	+	+	-
IR-901	2	-	+	-
CIM-446	2	+	-	-
CIM-240	3	+	-	-
BH-95	2	+	-	-
PB-900	4	+	-	-
BH-121	3	+	-	-
CIM-602	1	-	+	-
BP-S2-NC-63	1	-	+	-
VH-329	2	-	+	-
CIM1100	1	-	+	-
S-14	1	-	+	-
MNH-129	1	-	+	-
MNH-886	2	-	+	-
FH-183	2	-	+	-
KZ-189	2	-	+	-
Coker-307	2	-	+	-
CYTO-177	1	-	+	-
La Frego	4	+	-	-
B-557	1	-	+	-
CRIS-134	1	-	+	-
CIM-497	1	-	+	-
CIM-496	1	-	+	-
BIH-163	3	-	+	-
Gumbo Okra	3	-	+	-
Ukab 2/c	3	+	-	-
BH-121	3	+	-	-
FH-682	3	-	+	-
FH-142	2	+	-	-
MG-6	3	-	+	-
NH-268	2	+	-	-

Response of Cotton Germplasm to CLCuMuV, CLCuKoV-Bur and CLCuKoV

Many distinct species of begomoviruses are associated with CLCuD and exhibit the same kind of symptoms in the plants. By observing visual symptoms, it is not possible to identify particular virus species. All genotypes showed a differential response in PCR but most of the genotypes were found to be positive for Burewala strain and relatively less number of genotypes were positive for CLCuMuV (Table 3). Out of 38 genotypes, 26 (68%) were positive for Burewala strain and 13 (34%) were positive for CLCuMuV. This indicated that frequency of occurrence of Burewala strain was higher in the germplasm. The amplified product size by using these primers was 1760 bp for Burewala strain and 1740 bp for CLCuMuV (Fig. 3). All the genotypes were positive for Burewala strain or CLCuMuV, but we did not find any genotype, positive to CLCuKoV.

Varieties namely MNH-886 and FH-142, which are commercially cultivated on large areas of Punjab, are positive for Burewala strain and CLCuMuV, respectively. It means the parent species (CLCuMuV) and the recombinant strain (Burewala strain) both are prevailing in the fields in one growing season. Only 1 genotype named IR-6 showed scale of 5 in disease rating but it showed co-infection of both CLCuMuV and Burewala strain. Co-infection of different strains of CLCuD associated viruses in cotton is rare event but scale 5 shows that when there is a multiple infection, symptoms are very severe. Genotypes showing amplification to Burewala strain were rated from 1 to 3 in the disease scale. Interestingly, those cotton genotypes which were found to be positive for CLCuMuV showed disease severity scale between 2 to 5. This showed that CLCuMuV exhibited relatively more severe symptoms as compared to Burewala strain.

Discussion

Cotton leaf curl disease is a major biotic constraint for cotton production in Pakistan since the last two decades. After the occurrence of first epidemic in Pakistan, plant breeders are working for permanent and long lasting solution to develop resistance in high yielding varieties (Abbas *et al.*, 2015; Iqbal *et al.*, 2016).

Generally old screening methods are used to identify

Fig. 1: Symptomatic representation of disease scale of CLCuD used

A. Thickening of very few small veins on few leaves. B. Thickening of a small group of veins. C. Thickening of all veins. D. Severe vein thickening and leaf curling on the top third of the plant. E. Severe vein thickening and leaf curling on the half of the plant. F. Severe vein thickening, leaf curling, and stunting of the plant

Burewala strain. Burewala strain was most abundantly

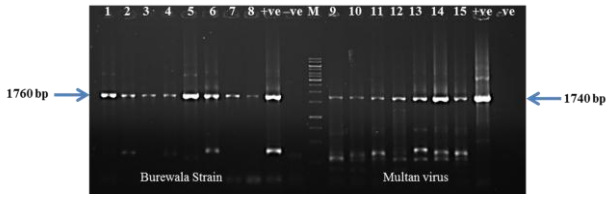

Fig 3: Lane 1-8 shows the 1760 bp sized product which is the result of PCR amplification by using the primer designed for Burewala strain, +ve control is the sequenced clone of Burewala strain, lane 9-15 shows 1740 bp sized product which is the result of PCR amplification by using the primer designed for cotton leaf curl Multan virus, +ve control is the clone of cotton leaf curl Multan virus. M is 1kb DNA Ladder

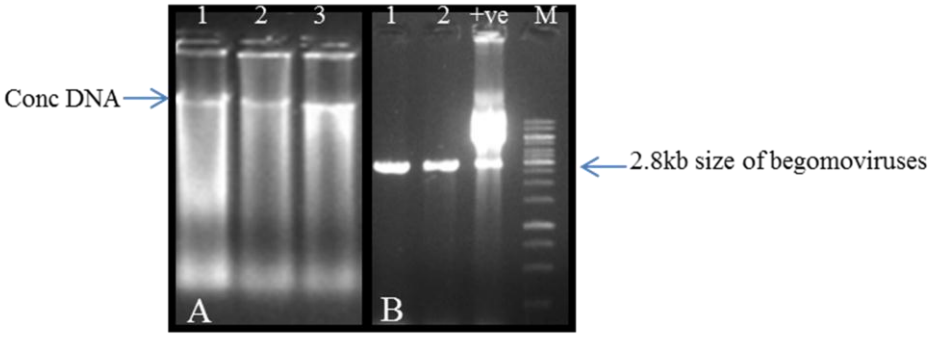

Fig. 2: Genomic DNA of cotton and PCR amplification of begomoviruses in cotton. A. DNA extracted by using CTAB method and run on 1% agarose gel. B. Lane 1 and 2 is amplification of cotton leaf curl disease associated viruses by using universal primers of begomoviruses. +ve control is a clone of cotton leaf curl Multan virus. M is 1kb DNA ladder (Fermentas)

the resistance sources from the germplasm, which can be further incorporated as parents in conventional hybridization programs. Lack of information about the species and strain, which are infecting the cotton crop in a particular season, is a big setback in cotton breeding programs. With the availability of molecular techniques, the molecular screening must be used along with the conventional approaches. Thus, the development of molecular tools may improve the breeding for resistance against viral diseases (Feuillet and Keller, 2005). The one step PCR has been used to identify viruses from plants and animals origin (Hu *et al*., 1993; Di *et al*., 2006; Liu *et al*., 2013). In this experiment, we developed a one-step diagnostic tool to identify species/strain, capable of infecting the cotton germplasm. Designing the species specific primers was cost effective and still more reliable than more laborious serological and host plant differential methods of virus identification. It will definitely help the plant breeders to screen their existing varieties.

Our results indicated that CLCuMuV and CLCuKoV-Bur are infecting the germplasm. However, some genotypes are susceptible to dual infection of CLCuMuV and spread in the germplasm. None of the genotypes were positive for CLCuKoV. This is an indication that CLCuKoV is no longer capable of infecting resistant cotton genotypes in Pakistan. Even though, CLCuKoV has been reported both in Pakistan and India from cotton plants (Sattar *et al.*, 2013), it appears that CLCuKoV was no longer capable of infecting our panel of resistant cotton varieties in Pakistan. Therefore, we expected that both the parent viruses and recombinant strains might co-occur at the same time. Surprisingly, only Burewala strain and CLCuMuV were found on cotton in our sample, which shows the natural selection of only two species infecting cotton varieties in Pakistan.

Conclusion

Our results demonstrate that strain specific PCR can be used to detect the viruses, infecting cotton germplasm in Pakistan. The primers designed in this study are very specific and can be used to faster the cotton breeding program. The PCR analysis revealed that one of the parental

strains of CLCuKoV-Bur is not prevalent in Pakistan, although CLCuMuV and CLCuKoV-Bur is widely prevalent in Pakistan.

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