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First Symptomatic Evidence of Infection of *Gossypium arboreum* with Cotton Leaf Curl Burewala Virus through Grafting

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Abstract

The resurgence of cotton leaf curl disease (CLCuD) since 2001, a major threat to cotton production in Pakistan is associated with Cotton leaf curl Burewala virus (CLCuBuV). All the available cultivated genotypes from *Gossypium hirsutum* at this time are susceptible however; the native cotton *G. arboreum* is believed to be immune to CLCuD. To investigate its exact resistance/susceptibility levels, young seedlings of genotype of *G. arboreum* (Ravi) and *G. hirsutum* (CIM-496) were graft inoculated with CLCuBuV under glass-house condition. All the graft-inoculated plants of *G. arboreum* (Ravi) and *G. hirsutum* (CIM-496) initiated disease symptoms 25 and 10 days respectively after inoculation. However, disease symptoms remained mild in *G. arboreum* genotype Ravi (resistant), while severe in case of *G. hirsutum* genotype CIM-496 (highly susceptible) throughout the experiment (90 days after graft-inoculation). All the graft-inoculated plants were found to be positive for CLCuBuV and cotton leaf curl Multan beta Burewala ($CLCuMB^{Bur}$) through PCR. This is the first symptomatic evidence of *G. arboreum* to be infected with virus causing CLCuD through grafting. The information generated under the present investigations would be helpful to provide better under-standing regarding the molecular basis of resistance to resolve this issue.

Keywords: Cotton; *G. arboreum*; Graft inoculation; Leaf curl disease; Symptomatic evidence

Introduction

Cotton (*Gossypium* spp.) also known as "white gold" is an economically important natural fiber crop as well as second most important oil seed crop in many warm areas of the world (Tuttle *et al.,* 2008; Iqbal *et al.,* 2011; Shah *et al.,* 2011). The genus *Gossypium* is comprised of approximately 45 diploid and five allotetraploid species (Fryxell, 1979). However, cultivated cotton is comprised of only four species viz; *Gossypium hirsutum* (allotetraploid, AD1), *G. barbadense* (allotetraploid, AD2), *G. arboreum* (diploid, A2) and *G. herbaceum* (diploid, A1) (Azhar *et al.,* 2011). In Indian subcontinent all these four species are grown successfully. The fact remains that the area under other species is just nominal as compared to *G. hirsutum*, which have high yield and excellent fiber traits (Chandra and Sreenivasan, 2011).

In Pakistan cotton, especially *G. hirsutum* is a high value crop. Since 1988 cotton leaf curl disease (CLCuD) has caused severe losses in the production by the emergence and reemergence of new virus strains (Akhtar *et al.,* 2008; 2009; 2010). More recently CLCuD had been associated with whitefly, *Bemisia tabaci* transmitted single begomovirus, designated as cotton leaf curl Burewala virus (CLCuBuV). Before 2001, the disease was found to be associated with a single type of DNA β satellite interacting with seven distinct begomviruses (Amrao *et al.,* 2010). The typical symptoms of CLCuD includes leaf rolling and smalling, vein darkening, production of leaf-like out-growths called "enations" and plant stunting (Akhtar *et al.,* 2010).

Development of resistant varieties is the only option to manage CLCuD. During the past, the efforts were made to breed CLCuD-resistant cultivars, but unfortunately introgressed host plant resistance was rapidly overcome by a resistance breaking strain during 2001 and all the available cultivated genotypes from *G. hirsutum* at this time are susceptible (Akhtar *et al.,* 2010). However, interestingly the native cotton *G. arboreum* also known as "Desi cotton" appears not to be infected with CLCuD till the first inception of disease (Rahman *et al.,* 2002; 2005). *G. arboreum* is believed to be evolved from its wild ancestor *G. herbaceum* (Hutchison *et al.,* 1984). *G. arboreum* possess many favorable agronomical, entomological and physiological attributes, which the *G. hirsutum* cultivars lack (Liu *et al.,* 2006). On the other hand, *G. arboreum* has poor fiber quality traits and does not fit for processing on modern processing machines (Chandra and Sreenivasan, 2011). However, utilization of disease resistant attributes from *G. arboreum* to *G. hirsutum* genotypes is the demand of time after the emergence and re-emergence of

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CLCuD in Indian subcontinent.

Study of resistance/susceptibility is believed to be rather difficult and laborious, because of the involvement of vector and the efficiency of transmission, acquisition period, persistence, and semi-persistent nature of viruses, as well as the host-vector virus interactions. However, these do not pose any problem in graft inoculated viruses. Grafting may succeed in transmission of a virus where other methods fail as it involves the union of cambial layers of stock and scion, either of which might be infected by a virus (Matthews, 1970; Akhtar *et al*., 2003).

G. arboreum is believed to be immune to CLCuD (Rahman *et al.*, 2005; Azhar *et al.*, 2011). Akhtar *et al*. (2010) observed the latent infection in case of *G. arboreum*, as they did not find any visible symptoms after graft inoculation with CLCuBuV. However, the exact status of its resistance level against CLCuD is still not clear and controversial. So to determine whether CLCuBuV can produce symptoms in *G. arboreum*, graft inoculation experiment was conducted under glass-house conditions.

Materials and Methods

Six-weeks old, five plants of each of the two genotypes of *G. arboreum* (Ravi) and *G. hirsutum* (CIM-496) were graft-inoculated separately with CLCuBuV following Akhtar *et al*. (2002). For graft-inoculation, a sliced cut was made with the help of a scalpel near the growing tip of test plant. A fresh branch with CLCuBuV symptoms was detached from glass-house maintained diseased plant and a similar cut was also made on this branch. Then cut surfaces were brought together and tied with parafilm. The symptomatic cut stem was then dipped in a test tube having distilled water. Distilled water was filled daily and after seven days the tubes were removed. Experimental unit was observed daily for grafting success, disease transmission and symptom development; latent period and disease severity till the end of experiment (90 days after grafting) using the disease scale described by Akhtar *et al*. (2010).

Both symptomatic and non-symptomatic leaves were collected from all the graft inoculated plants of the both test varieties 60 days after grafting. Total genomic DNA was isolated from these leaves by cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Proper dilution of DNA was prepared and was subjected to rolling circle amplification (RCA) using Phi-29 DNA polymerase. Phi amplified DNA was subjected to PCR for the amplification of cotton leaf curl Multan beta Burewala ($CLCuMB^{Bur}$) and cotton begomovirus using specific primers (Briddon *et al*., 2002; Amrao *et al.*, 2010).

Results

Success of grafting was 100% and all the grafted scions survived till the end of the experiment. Disease transmission was also 100% as all the graft-inoculated plants of *G. arboreum* (Ravi) (Fig. 1) and *G. hirsutum* (CIM-496) developed CLCuD symptoms with wide variation in severity index (SI) and latent period (LP) values (Table 1). RCA done for the samples collected from inoculated plants showed positive amplification (results not shown). This RCA amplified DNA was then subjected to PCR using $CLCuMB^{Bur}$ specific primers and CLCV Begomo F, Begomo R primer for the amplification of betasatellite and begomovirus components, respectively. Positive amplification was seen in all the samples both for betasatellite (1.4kb) and begomovirus (1.1 kb for Begomo diagnostic CLCV primers and 2.8 kb for full length Begomo primers; Fig 2a, b and c).

G. hirsutum genotype CIM-496 was the first to develop disease symptoms 10 days post inoculation and showed sever symptom including leaf rolling, veins darkening and "enations" (Fig. 3a) with SI value of 6E (highly susceptible) within 20-25 days post inoculation with no reduction in disease severity through out the experiment (90 days after graft-inoculation). Flowers and bolls on these plants were reduced with veins swelling/darkening and "enations" on bracts (Fig. 3b). However, all the graft-inoculated plants of *G. arboreum* (Ravi) initiated disease symptoms 25 days after inoculation, with SI value 1E (resistant). CLCuD-symptoms on *G. arboreum* genotype Ravi were started as slight vein swelling/darkening; some of these were later developed into enations (Fig. 4a and b). These types of symptoms were developed on 3–10% leaves of all graft-inoculated plants of *G. arboreum* (Ravi). CLCuBuV and $CLCuMB^{Bur}$ were readily detected in both symptomatic and symptom-less leaves of these plants. However, the pattern of symptom expression varied and symptom development was found to be initiated from lower older leaves (present above the grafted points), whereas newly emerging leaves were symptom-less. Symptomatic plants of *G. arboreum* (Ravi) also showed minor shortening of internodes and reduction in plant height as compared to healthy plants (results not shown). Nevertheless, no increase in the SI value of *G. arboreum* genotype Ravi was observed till the end of the experiment, as plants showed no symptoms on upper half of the plant. Symptom-less leaves of all the graft inoculated plants of *G. arboreum* (Ravi) were also found to be positive for CLCuBuV and $CLCuMB^{Bur}$.

Discussion

This is the first published evidence on the unexpected production of CLCuD symptom phenotypes in *G. arboreum* due to CLCuBuV through grafting. In most studies, *G. arboreum* is thought to be immune (Azhar *et al.*, 2011) and non-host against CLCuD. The results presented here demonstrated that *G. arboreum* is infected with CLCuBuV and is the host of CLCuBuV. As the term immunity for host plant resistance refers to a non-host response that is manifested by the inability of a particular plant species (Torp and Jorgenson, 1986; Rahman *et al*. 2005).

Table 1: Graft inoculation results of *Gossypium* species against CLCuV under glasshouse conditions

Gossypium spp./(genotypes)	Plant No.	Grafting success*	Disease transmission	Latent period (Days)	Disease severity 90 days post inoculation	CLCuBuV detection through PCR	CLCuMB^{Bur} detection through PCR	Disease response
G. arboreum (Ravi)	1	+	+	26	1E	+	+	Resistant
	2	+	+	25	1E	+	+	Resistant
	3	+	+	24	1E	+	+	Resistant
	4	+	+	24	1E	+	+	Resistant
	5	+	+	26	1E	+	+	Resistant
Average		100 %	100 %	25	1E **	100 %	100 %	Resistant
G. hirsutum (CIM-496)	1	+	+	10	6E	+	+	Highly susceptible
	2	+	+	10	6E	+	+	Highly susceptible
	3	+	+	10	6E	+	+	Highly susceptible
	4	+	+	10	6E	+	+	Highly susceptible
	5	+	+	10	6E	+	+	Highly susceptible
Average		100 %	100 %	10	6E **	100 %	100 %	Highly susceptible

*Success of grafting was "+" when grafted stem (scion) survived for more than 20 days after grafting
Note that grafting was successful till the end of the experiment
**Severity index (SI)

Fig. 1: Picture showing union of the grafted root stock of *G. arboreum* genotype Ravi and scion of the symptomatic susceptible *G. hirsutum* genotype CIM-496

Disease transmission was 100% on all the successfully grafted plants of *G. arboreum* (Ravi) and *G. hirsutum* (CIM-496) in the present case. Positive amplification was seen in all the samples both for CLCuBuV and CLCuMB^{Bur} through PCR. As expected *G. hirsutum* genotype CIM-496 was the first to develop disease symptoms 10 days after graft-inoculation and showed sever symptom within 20-25 days post inoculation with no reduction in disease severity till the end of the experiment. Conversely, all the graft inoculated plants of *G. arboreum* (Ravi) unexpectedly initiated disease symptoms 25 days after graft-inoculation as slight vein swelling/darkening and enations on 3–10% leaves. These findings are in contradict with those of Rahman *et al*. (2002, 2005) who found that *G. arboreum* cannot be infected with CLCuD and cannot develop symptoms even under heavy loads of virus through grafting. Akhtar *et al*. (2010) also observed the latent infection in case of *G. arboreum* (Ravi), as they did not find any visible symptoms after graft-inoculation with CLCuBuV, but found

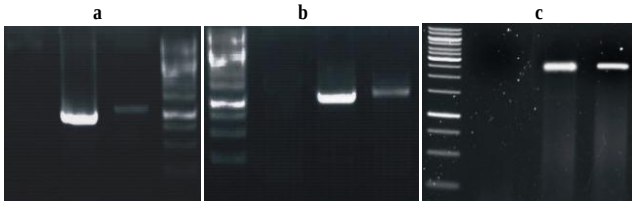

Fig 2: (a) PCR amplification of *Gossypium arboreum* samples using CLCuMB^{Bur} primers. In the fig negative control (lane 1), Positive control (lane 2), *G. arboreum* (lane 3) and 50bp marker (lane M); **(b)** PCR amplification of *G. arboreum* samples using CLCV1 and CLCV2 primers. In the fig negative control (lane 1), Positive control (lane 2), *G. arboreum* (lane 3) and 1kb marker (lane M) and **(c)** PCR amplification of *G. arboreum* samples using Begomo F and Begomo R primers. In the fig negative control (lane 1), Positive control (lane 2), *G. arboreum* (lane 3) and 1kb marker (lane M)

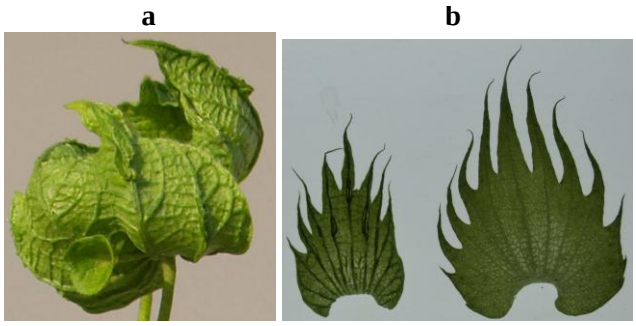

Fig. 3: (a) *Gossypium hirsutum* susceptible genotype showing severe vein thickening, leaf rolling and enations and **(b)** bract from infected flower showing severe vein thickening and enations on left & from healthy on right

80% plants positive for CLCuBuV through PCR. We followed the same experimental conditions as used by Akhtar *et al*. (2010), except for slight fluctuation in minimum and maximum temperature ranges due to the unexpected changes in weather conditions out side the

Fig. 4: (a) *Gossypium arboreum* showing minor vein swelling and darkening and **(b)** *G. arboreum* showing vein enation

glasshouse. We hypothesized that this may be one of the reasons of contradictory results. Because environmental factors such as temperature is found to have great influence on plant virus interactions (Szittya *et al.,* 2003; Chellappan *et al.,* 2005; Tuttle *et al.,* 2008; Ogwok *et al.,* 2010). However, there is no obvious expiation for this and it needs further probe.

This study also reported that symptom development in all the graft-inoculated plants of *G. arboreum* (Ravi) was found to be initiated from lower older leaves (present above the grafted points) contrary to *G. hirsutum* genotype (CIM-496), in which symptoms were first developed in newly emerging leaves. Although, CLCuBuV and CLCuMB^{Bur} were readily detected in both symptomatic and symptom-less leaves collected from different locations of all these graft-inoculated *G. arboreum* (Ravi) plants. This might be due to the higher accumulation of virus particles in older leaves as compared to younger leaves, as earlier reported for cassava brown streak virus (Ogwok *et al*., 2010). Further studies are in progress to prove this through southern blotting and quantitative real-time PCR.

In conclusion, this is the first published evidence on the surprising production of CLCuD symptom in *G. arboreum*. Present study also proved that *G. arboreum* is not immune but is host against CLCuV. *G. arboreum* is thought to have some inherent extraordinary agronomic, entomological, pathological and physiological traits, a good source of resistance against CLCuV and is suitable for breeding programs. This information will improve our understanding on the molecular basis of resistance.

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