EXAMINATION BY GRAFTING OF THE EXTREME RESISTANCE TO COTTON LEAF CURL DISEASE IN TWO WILD *GOSSYPIUM HIRSUTUM* L. CULTIVARS

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SUMMARY

Cotton leaf curl disease (CLCuD) is a serious threat to cotton production across the Indian subcontinent. In the Punjab province of Pakistan the disease is associated with a distinct strain, the Burewala strain, of cotton leaf curl Kokhran virus (CLCuKoV-Bu) and a distinct strain of the symptom-determining betasatellite, cotton leaf curl Multan betasatellite-Burewala (CLCuMuB^{Bur}). At this time all commercial varieties of *Gossypium hirsutum* are susceptible to CLCuD. Two exotic *G. hirsutum* wild accessions, AS0039 and AS0099 from Caribbean, were found to be naturally resistant to CLCuD. However, the nature of the resistance has remained undefined. Graft-inoculation and whitefly transmission assays were conducted as a preliminary analysis of the resistance of these cultivars to CLCuKoV-Bu/CLCuMuB^{Bur} complex. By whitefly transmission under conditions where all plants of the susceptible cultivar CIM-496 became symptomatic AS0039 and AS0099 plants remained non-symptomatic and no virus was detected. Graft-inoculation of AS0039 and AS0099 with severely infected scions from *G. hirsutum* cv. CIM-496 showed that these accessions could support the replication and long-distance spread of the virus/betasatellite-complex. Several leaves developing at the time of grafting close to and distal to the grafts in these plants showed mild symptoms with low amounts of viral/betasatellite DNA; significantly less than the levels seen in susceptible CIM-496 plants. Although symptoms spread to leaves away from the initial point of infection, the youngest leaves at the apex of both AS0039 and AS0099 plants remained symptomless with very low virus/betasatellite-complex. The results showed that the resistance of AS0039 and AS0099 plants is either due to reduced virus/satellite replication or perhaps interference in the short distance (cell-to-cell) spread of the virus/betasatellite-complex.

Keywords: Host-plant resistance, exotic accessions, *Begomovirus*, cotton leaf curl disease, cotton leaf curl Kokhran virus – Burewala.

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INTRODUCTION

Cotton leaf curl disease (CLCuD) is a threat to cotton production across the world and has recently been introduced into southeastern China (Sattar *et al.*, 2013). The disease is the major biotic constraint to the production of cotton across Pakistan and north-western India and is caused by *Bemisia tabaci*-transmitted viruses of the genus *Begomovirus* (family *Geminiviridae*; Khan *et al.*, 2016). First reported as a sporadic nuisance in 1967 in Multan (Pakistan), the disease became epidemic for the first time in 1989 and was shown to be associated with four distinct monopartite begomovirus species, the most important of which were cotton leaf curl Multan virus (CLCuMuV) and cotton leaf curl Kokhran virus (CLCuKoV; Brown *et al.*, 2015), as well as a symptom-modulating betasatellite, cotton leaf curl Multan betasatellite (CLCuMuB; Briddon *et al.*, 2001). During late 1990s CLCuD-resistant *G. hirsutum* lines, introduced through conventional breeding programs utilizing both indigenous as well as exotic germplasm such as CP-15/2 and LRA-5166, overcame losses from the disease (Akhtar *et al.*, 2008). However, this resistance proved temporary and the disease soon reappeared in a resistance-breaking form in 2001-02 near the city of Burewala (Pakistan). This signaled a second epidemic of the disease spreading throughout Pakistan and into north-western India. CLCuD post-resistance breaking was shown to be associated with a single begomovirus, the "Burewala" strain of CLCuKoV (CLCuKoV-Bu; earlier known as cotton leaf curl Burewala virus), and a recombinant CLCuMuB (CLCuMuB^{Bur}; Amrao *et al.*, 2010). The precise mechanism of resistance breaking by CLCuKoV-Bu/CLCuMuB^{Bur} remains unclear, although the genetic changes in the virus and betasatellite are yielding some clues to this (Briddon *et al.*, 2014).

The genus *Gossypium* encompasses *ca.* 50 species that include the leading source of natural fiber (Campbell *et al.*, 2009). The species are grouped into eight genome types with distinct evolutionary histories (Wendel *et al.*, 2009). Of the four species producing spin able fibers, two are diploid (*G. arboreum* and *G. herbaceum*) with an Asiatic origin and two are tetraploid (*G. hirsutum* and *G. barbadense*),

having their origins in the Americas. Dominating worldwide cotton production, *G. hirsutum* has replaced the cultivation of the other three species in most counties, producing over 90% of the cotton annually (Wendel and Cronn, 2003; Siddique *et al*., 2015). The adaptation of the tetraploid *G. hirsutum* to grow in wide-ranging conditions, however, has brought new challenges that include emergent problems with pests and diseases (Mansoor and Paterson, 2012).

Although the diploid cotton species possess desirable attributes such as resistance to abiotic and biotic stresses, including resistance to CLCuD in particular (Siddique *et al*., 2014; Ullah *et al*., 2014), the introgression of these traits into *G. hirsutum* is complicated by differing ploidy levels between the two species. Despite these problems there have been recent successes in transferring CLCuD resistance from *G. arboreum* to *G. hirsutum* (d'Eeckenbruggeand Lacape, 2014). Nevertheless, all currently available commercial *G. hirsutum* lines are susceptible to CLCuD and the disease continues to cause significant crops losses (Akhtar *et al*., 2010, 2015).

In the search for new sources for CLCuD-resistance, *G. hirsutum* cvs AS0039, originating from Dominica, and AS0099, originating from Haiti, were imported from Caribbean. These cultivars were shown to be highly resistant to CLCuD in contained whitefly-mediated inoculation screening (Akhtar *et al*., 2010). The study described here provides detailed analysis of the nature of resistance in these accessions and shows the resistance to be suitable for use in the field to fight CLCuD-causing begomoviruses.

MATERIALS AND METHODS

Whitefly transmission assays. Non-viruliferous adult whiteflies were reared on *G. arboreum* cv. Ravi plants in an insect-free glasshouse. These adult whiteflies were allowed a *ca.* 72 hours (h) acquisition access period (AAP) on greenhouse-maintained graft-inoculated CIM-496 plants severely infected with the CLCuKoV-Bu/CLCuMuB-complex (accession Nos. HF569171 and HF912232). Fifteen plants of each accession (exotic wild *G. hirsutum* cvs AS0039 and AS0099) were grown in a glasshouse. One susceptible genotype CIM-496 was also included as positive control. At the age of 4-5 weeks these pots for each genotype were divided into three groups. Each group, comprising five pots, was covered with muslin cloth under iron cages. Then each group for each genotype was exposed to 50, 100 and 150 viruliferous whiteflies per plant for an inoculation access period (IAP) of 72 h. Following the IAP the whiteflies were killed using the insecticide Confidor (Bayer), the plants were moved to an insect-free glasshouse and monitored for the appearance of symptoms. The experimental unit was observed daily for the appearance of disease symptom and data were collected following the rating system described by Akhtar *et al.* (2015).

Graft-inoculation assays. Six-week-old plants of each test genotype maintained in insect-free glasshouse were graft inoculated (20 of each in 2011 and 2012) following an improved "bottle shoot" grafting technique (Akhtar *et al*., 2002; Ullah *et al*., 2014). The scions for grafting were obtained from severely infected, glasshouse-maintained *G. hirsutum* cv. CIM-496 plants. The graft-inoculated unit was maintained by following normal agronomic practices in large earthenware pots in an insect-free glasshouse with a day-time temperature between 38°C and 45°C and a night-time temperature between 25°C and 30°C. The experimental unit was observed daily and data were recorded on the percentage of successful grafts, percentage of disease transmission, latent period (average time required for first symptom appearance after grafting) and SI 180 days post inoculation (DPI) using the rating system described by Akhtar *et al.* (2015).

Virus/betasatellite detection and characterization. DNA was extracted from leaf samples using the CTAB method (Doyle and Doyle, 1990). Molecular diagnostics for the presence of virus and betasatellite was performed using primer pairs CLCV1/CLCV2 (Ullah *et al*., 2014) and Beta01/Beta02 (Briddon *et al*., 2002) to detect begomoviruses and betasatellites, respectively. Circular DNA molecules in DNA samples extracted from plants were amplified by rolling circle amplification (RCA; Haible *et al*., 2006) according to the manufacturer's instructions (Thermo Fisher Scientific GmbH, Germany). Full length begomoviruses and betasatellites were amplified from RCA products by PCR using primer pairs Begomo-F/Begomo-R (Shahid *et al*., 2007) and Beta01/Beta02, respectively. PCR-amplified products were purified and cloned into the pTZ57R/T vector (Thermo Fisher Scientific GmbH, Germany). The inserts of clones were sequenced commercially (Macrogen, South Korea). Sequences were assembled using SeqMan, part of the Lasergene sequence analysis package (DNAStar, USA), and compared with sequences available in the databases by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Southern blot hybridization of DNA samples from leaf tissues was performed following the method described previously (Ullah *et al*., 2014). Extracted DNAs from plants leaves, electrophoresed on 0.8% (w/v) agarose gels, were blotted onto positively charged nylon membranes and UV cross-linked. Digoxigenin-labelled virus and betasatellite specific probes, using primers pairs CLCV1/CLCV2 (Ullah *et al*., 2014) and Beta01/Beta02 (Briddon *et al*., 2002), respectively, were prepared using DIG DNA labelling kit (Thermo Fisher Scientific GmbH, Germany). Hybridization, followed by high stringency washings, was performed at 55°C for 12-16 h and after treatment with CDP-Star (Thermo Fisher Scientific GmbH, Germany) the signals were detected on X-ray films.

RESULTS

Disease response after whitefly transmission of virus. For CIM-496 plants caged with 50 insects the first symptoms appeared 14 days post inoculation (dpi). For CIM-496 plants caged with either 100 or 150 insects the latent period was shorter (11 dpi). The initial symptoms in CIM-496 plants consisted of minor vein thickening and darkening, which ultimately developed into full CLCuD symptoms consisting of leaf curling both upward and downward, vein thickening and darkening, the formation of enations and stunted plant growth for all 15 plants (results not shown). In contrast, none of the AS0039 or AS0099 plants developed any symptomatic evidence of the disease over the six month period after whitefly inoculation. This experiment was conducted twice, in 2011 and 2012, with the same results.

PCR diagnostics using primer pairs CLCV1/CLCV2 and Beta01/Beta02 yielded PCR products of *ca.* 1100 nt and *ca.* 1355 nt for the 15 CIM-496 at 15, 30, 60, 90, 120 dpi and at the end of the experiment (180 dpi). In contrast, no amplification was obtained at any time point for AS0039 or AS0099 plants, even when the extracted DNA samples were first enriched for circular DNA molecules by RCA (so called RCA/PCR).

Disease response upon graft-inoculation of virus. A total of 40 plants each of AS0039 and AS0099 (20 of each in 2011 and 2012) were graft-inoculated with scions from greenhouse-maintained graft-inoculated CIM-496 plants with severe symptoms of CLCuD. Of these, 15 (75%) and 17 (85%) of the AS0039 plants and 13 (65%) and 16 (80%) of the AS0099 plants, in 2011 and 2012, respectively, developed mild symptoms of CLCuD. The first symptoms of infection became evident at *ca.* 25 days post-grafting for AS0039 and 26-27 days post-grafting for AS0099. For these cultivars the first symptoms developed relatively late in comparison to the susceptible cultivar CIM-496 in which the first symptoms of infection appeared 9-10 days post-grafting. The initial symptoms in all graft-inoculated plants (AS0039, AS0099 and CIM-496) appeared as a slight vein thickening and darkening. For CIM-496 plants these initial symptoms developed into severe CLCuD symptoms with leaf curling and cupping, vein swelling and darkening, and the formation of enations from undersides of the leaves (Fig. 1a). In contrast, the symptoms for AS0039 and AS0099 plants remained mild, consisting of mild swelling and some darkening of the primary, secondary and tertiary veins (Fig. 1b, c). The symptoms for AS0039 and AS0099 initiated on the leaves that were developing at the time of grafting on the branch immediately above the graft (branch 1 in Fig. 2) and subsequently on leaves on the branches above that (branches 2 and 3 in Fig. 2). However, the top most leaves (indicated as branch 4 in Fig. 2) in all of the graft-inoculated AS0039 and AS0099 plants remained symptomless.

Southern blot analysis of DNA samples extracted from symptomatic leaves (indicated as branches 1, 2 and 3 in Fig. 2) of graft-inoculated AS0039 and AS0099 plants (40 days after the first appearance of symptoms) showed weak hybridization signals with both virus- and betasatellite-specific probes (Fig. 3). The levels of hybridization in both AS0039 and AS0099 plants were significantly lower than those of graft-inoculated CIM-496 plants, suggesting very low virus and betasatellite DNA levels. Replicative forms of DNA, suggestive of virus replication, were evident in samples from both AS0039 and AS0099 plants. Hybridization signals were not detected in samples extracted from non-symptomatic leaves of graft-inoculated AS0039 and AS0099 plants (the top most leaves; indicated as branch 4 in Fig. 2) or in any of the control, non-grafted AS0039 and AS0099 plants.

Prior to grafting, DNA samples from all of the AS0039 and AS0099 plants were found to be uniformly negative for the presence of virus and betasatellite by PCR and RCA/PCR diagnostics. However, both the infected scions and the symptomatic leaves of AS0039 and AS0099 plants after successful grafting (labelled as branches 1, 2 and 3 in Fig. 2) were found to be positive for the presence

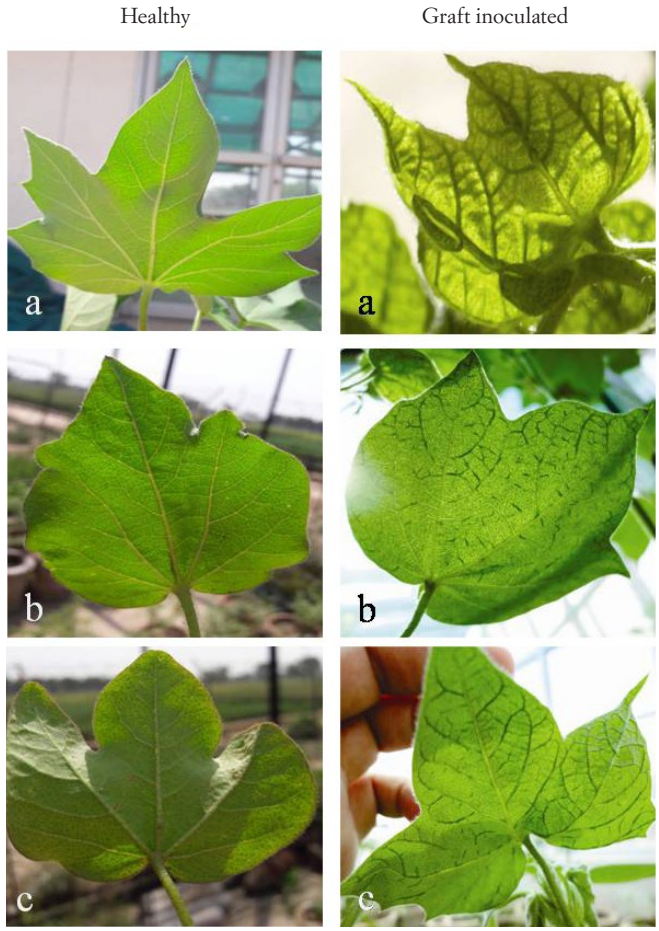

Fig. 1. Foliar symptoms of graft-inoculated susceptible CIM-496 (a) and resistant AS0039 (b) and AS0099 (c) plants. The photos are of leaves of branch 1 in Fig. 2.

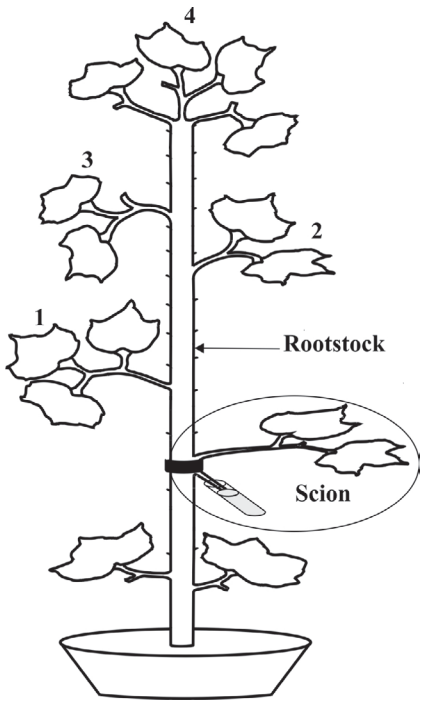

Fig. 2. Diagrammatic representation of the graft-inoculation of AS0039 and AS0099 and susceptible control CIM-496 (labeled rootstock) using scions from infected CIM-496 plants. The rootstock (representing AS0039, AS0099 and CIM-496) and the infected/symptomatic scion from CIM-496 are shown. The tube of water, used to prevent the graft from wilting whilst a union is established, was removed at 7-9 days after grafting. The branches, indicated as 1 to 4, are discussed in the text.

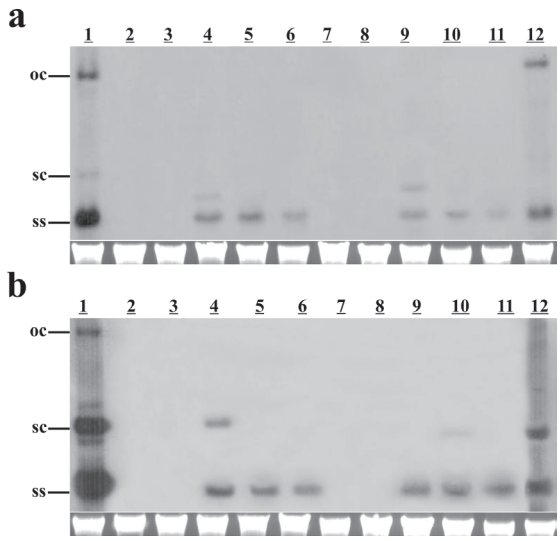

Fig. 3. Southern blot hybridization of DNA samples extracted from the leaves of cotton plants probed for the presence of cotton leaf curl Kokhran virus - Burewala (panel a) and cotton leaf curl Multan betasatellite (panel b). The DNA samples were extracted from the graft (graft-inoculated symtomatic CIM-496 used as scion; lanes 1 and 12 in both blots), healthy non-graft-inoculated AS0039 and AS0099 plants (lanes 2 and 7, respectively, in both blots), symptomatic leaves of graft-inoculated AS0039 and AS0099 plants collected 40 days after appearance of the first symptoms of the disease (lanes 4, 5 and 6, and lanes 9, 10 and 11, respectively, in both blots; branches 1, 2 and 3, respectively, on Fig. 2), non-symptomatic leaves (branch 4 on Fig. 2) of graft-inoculated AS0039 and AS0099 plants collected 40 days after appearance of the initial symptoms of the disease (lanes 3 and 8, respectively, in both blots).

of virus and betasatellite by PCR. However, only RCA/PCR of DNA samples from the upper leaves (indicated as branch 4 in Fig. 2) of graft-inoculated AS0039 and AS0099 plants detected the presence of both virus and betasatellite. PCR without prior RCA was not successful in detecting virus and betasatellite in any DNA sample from the upper leaves of graft-inoculated AS0039 and AS0099 plants. RCA/PCR diagnostic with samples extracted from healthy, non-grafted AS0039 and AS0099 plants were also uniformly negative.

Response of wild accessions to viral infection upon removal of the graft inoculated diseased scion. The mildly symptomatic AS0039 and AS0099 plants were chosen for further analysis of the infection. At *ca.* 5-10 days after the appearance of the initial symptoms, the grafts (scions) were removed from 7 and 8, and 6 and 7 of the AS0039 and AS0099 plants, respectively. The remaining plants with successful grafting were maintained as controls for comparison. A systemic spread of the disease symptoms from the initial point of infection to the next leaves (indicated as branches 2 and 3 in Fig. 2) was observed in all of the control plants in which the grafts were maintained over a period of more than eight weeks. The newly emerging leaves at the top (indicated as branch 4 in Fig. 2) in all of the controls remained symptomless. On the other hand, the systemic spread of the disease symptoms from the initial point of infection to the newly emerging leaves ceased in all of the AS0039 and AS0099 plants from which the grafts had been removed. In graft-removed plants the spread of symptoms was seen in some leaves on branch 2 following the initial point of infection (branch 1, at the time of removing the grafts), which were very mild in comparison to the control plants for which the grafts were maintained (results not shown). Both virus and betasatellite were amplified by RCA/PCR from the topmost leaves in all of the control, graft-maintained AS0039 and AS0099 plants but not from graft-removed plants.

Back-indexing to CIM-496 plants. The mildly symptomatic branches from graft-inoculated AS0039 and AS0099 plants were grafted back onto 10 CIM-496 plants each. The grafting was conducted twice, in 2011 and 2012. Of the 10 graft-inoculated CIM-496 plants 6 and 8 (with scions originating from AS0039), and 7 and 8 plants (with scions originating from AS0099) in 2011-12, respectively, showed initial symptoms of infection at 9-12 days post-grafting. The symptoms started as mild vein thickening and darkening in the newly emerging leaves that increased in severity to give severe CLCuD symptoms (results not

shown). Seven graft-inoculated CIM-496 plants did not develop any visible CLCuD-symptoms due to the lack of successful graft union in these plants. Four graft-inoculated CIM-496 plants, despite apparently developing a successful graft union, remained symptomless with reason remaining unclear. All the symptomatic graft-inoculated CIM-496 plants were found to be positive for the presence of virus/betasatellite-complex each time they were tested throughout the experiment. However, all of the healthy/non graft-inoculated along with graft-inoculated non-symptomatic CIM-496 plants showed no amplification of virus/betasatellite-complex by PCR and RCA/PCR.

Confirmation of the transmitted virus/betasatellite. Begomoviruses and their cognate betasatellites were amplified by PCR using the universal primer pairs Begomo-F/Begomo-R and Beta01/Beta02, respectively, and cloned. A total of 8 begomovirus clones were obtained and sequenced in their entirety; 4 from infected CIM-496 plants which were used as scions for grafting (accession Nos. HF952157 and HG428705 in 2011, and LK995396 and LK995397 in 2012) and 4 from symptomatic leaves of graft-inoculated AS0039 and AS0099 plants used as the rootstocks (HF952154 and HG428704 from AS0039, and HF952155 and HG428706 from AS0099, in 2011 and 2012, respectively).

Nucleotide BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of all 8 full-length clones showed the highest level of nucleotide sequence identity (99-100%) to isolates of CLCuKoV-Bu available in the database. Pairwise sequence comparisons, by Sequence Demarcation Tool (SDT) v. 1.0 using MUSCLE alignment (http://web.cbio.uct.ac.za/SDT), confirmed these clones to be isolates of CLCuKoV-Bu according to the revised classification of begomoviruses. When analyzed using ORF finder, all clones showed the lack of a full-length *C2* gene (results not shown) which is the unique feature of CLCuKoV-Bu associated with resistance breaking.

A total of 8 betasatellite clones were also obtained and sequenced in their entirety; 4 from CIM-496 plants which were used as scions for grafting on AS0039 and AS0099 (accession Nos. HF952156 and LK995398, and HG428707 and LK995399 in 2011 and 2012, respectively) and 4 from symptomatic leaves of graft-inoculated AS0039 and AS0099 plants (HF952152 and HG428703, and HF952153 and HG428702 in 2011 and 2012, respectively). Nucleotide BLAST analysis showed the highest levels of nucleotide sequence identity (89.2-99%) of these clones to isolates of CLCuMuB. Furthermore an alignment to earlier determined CLCuMuB sequences showed the isolates here to have the insertion from another betasatellite, tomato leaf curl betasatellite, which is typical of the strain of CLCuMuB associated with resistance breaking.

DISCUSSION

Throughout the experiments described here the nature of the virus being transmitted was monitored and shown to be CLCuKoV-Bu with the recombinant form of CLCuMuB (CLCuMuB^{Bur}). This virus/betasatellite complex has been the major, if not the only, virus or virus complex causing CLCuD across much of Pakistan since the early 2000s, when resistance in cotton was first broken (Mansoor *et al.*, 2003). There can thus be no doubt that the response to virus inoculation of cvs AS0039 and AS0099 is to the resistance-breaking complex.

The studies conducted showed that both AS0039 and AS0099 are not susceptible to CLCuKoV-Bu/CLCuMuB-^{Bur} by insect transmission as no virus or betasatellite was detected in the plants of these accessions even after using the most sensitive diagnostic technique (RCA/PCR) available at this time. The high level of resistance to CLCuKoV-Bu/CLCuMuB^{Bur} by insect transmission in these accessions could be because of the resistance to the vector *B. tabaci*. However, it may also be the result of an inability of whitefly to introduce a sufficient quantity of virus particles into the plants to cause a disease (Akhtar et al., 2010). However, disease transmission programmes that rely on vector inoculation may produce ambiguous results as the vector resistance can be interpreted as resistance to the virus. This difficulty can be overcome by some more efficient artificial inoculation methods like *Agrobacterium*-mediated inoculation or biolistic inoculation to introduce cloned viruses into cotton plants. Though, these methods were unsuccessful to introduce cloned viruses into cotton plants. So, in the absence of a such type inoculation method, grafting provides an efficient means of introducing virus without the need for a vector. Therefore, an improved bottle shoot graft inoculation method was used in the study described here to investigate the resistance to the viruses causing CLCuD. This method was previously used to investigate the resistance of *G. arboreum* to CLCuKoV-Bu and CLCuMuB^{Bur} (Akhtar *et al.*, 2013; Ullah *et al.*, 2014). By grafting, both AS0039 and AS0099 proved tolerant to CLCuKoV-Bu/CLCuMuB^{Bur} with plants developing mild symptoms of CLCuD. These findings suggest that a mechanism that affects the delivery of the virus to the plant by the vector insect is unlikely to be a major factor in the resistance of AS0039 and AS0099 to CLCuKoV-Bu/CLCuMuB^{Bur}. The major difference between graft inoculation and whitefly inoculation is in the amount of virus that is delivered to the plant. Arthropod vectors, such as *B. tabaci*, deliver only tiny amounts of virus in an intermittent and dispersed manner; each vector insect delivering virus at a different site and time. Caciagli and Bosco (1997) showed that *B. tabaci* insects contain at most 1.6 ng of the monopartite begomovirus tomato yellow leaf curl virus (TYLCV), and only a small portion of this will be delivered to a plant during a feed. This contrasts with graft inoculation which delivers virus in a continual

manner and in larger amounts. The results presented here are thus an indication that AS0039 and AS0099 have a high threshold level for establishment of (symptomatic) CLCuKoV-Bu/CLCuMuB^{Bur} infection, higher than that of the susceptible cultivar CIM-496. Such a dose-dependent threshold for infection has been shown for other virus-host systems and has been shown to be under genetic control (Collmer *et al*., 2000; Petty *et al*., 1990).

The results presented in this study show that resistance of AS0039 and AS0099 to CLCuKoV-Bu/CLCuMuB^{Bur} likely does not involve a mechanism that interferes with virus inoculation to the plant by the insect vector. Nor does the resistance appear to affect the long-distance spread of the virus in the phloem. Instead, the resistance of AS0039 and AS0099 appears to interfere with either virus replication or with local cell-to-cell movement of the virus. Both these mechanism would explain the lower levels of viral DNA in grafted AS0039 and AS0099 plants relative to the levels detected in CIM-496 plants. A number of resistance genes that interfere with virus replication have been identified such as JAX1 encoded by several ecotypes of *Arabidopsis thaliana* that interfere with the replication of potexviruses (Yamaji *et al*., 2012) and the tomato Tm-1 protein inhibits the replication of tobamoviruses (Ishibashi and Ishikawa, 2014).

Although both AS0039 and AS0099 are able to support the replication of the CLCuKoV-Bu/CLCuMuB^{Bur} complex, as long as there is a continual supply of virus from the graft, upon removal of the graft plants recover with the initially symptomatic leaves remaining symptomatic but all newly emerging leaves being asymptomatic and negative for virus and betasatellite. This would seem to suggest that the levels of virus/satellite replication in AS0039 and AS0099 tissues are insufficient to maintain a symptomatic infection, meaning that the virus complex is either lost or reduced below the detection levels. As previously concluded for the resistance of *G. arboreum* to CLCuKoV-Bu/CLCuMuB^{Bur} (Ullah *et al*., 2014), cotton cvs AS0039 and AS0099 have a resistance to CLCuKoV-Bu/CLCuMuB^{Bur} that is characterized by a high threshold for infection and appears to act by limiting either virus/satellite replication or cell-to-cell spread. The resistance of cvs AS0039 and AS0099 is, in the short term, likely to prove more useful than the resistance of *G. arboreum* since the resistance can be transferred without the problem of ploidy.

At this time, there are no commercially available cotton varieties/germplasm that are resistant to CLCuKoV-Bu/CLCuMuB^{Bur} (Akhtar *et al*., 2010, 2015). Cotton is a global commodity and a major foreign exchange earning crop for Pakistan and India. It is feared that the disease could spread to areas where, at present, it does not occur but where conditions are suitable for its proliferation; meaning just about anywhere where cotton is grown (Sattar *et al*., 2013). This possibility has been brought into stark contrast by the recent identification of CLCuD in China (Cai *et al*., 2010; Du *et al*., 2015). The resistant cultivars characterized here are a good source of resistance against CLCuD because these might affect the efficiency of vector-mediated transmission which constitutes the first barrier against the virus as previously reported by Delatte *et al*. (2006) in the case of TYLCV and should be introgressed into local germplasm at the earliest opportunity. However, learning a lesson from the earlier failure of resistance that was based on a single mechanism of action (a single gene) in the 1990s, possible early loss of the resistance due to evolution of resistance- breaking variants of the virus complex can most likely be avoided by pyramiding with other resistance genes, such as that recently identified in a collaboration between Pakistani and American plant breeders (http://www.ars.usda.gov/Research/docs.htm?docid=25107). It would also be wise to maintain the resistance introduced in the 1990s to avoid the return of the pre-resistance breaking CLCuD virus complex.

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