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RESEARCH ARTICLE

Molecular mapping of CLCuD resistance introgressed from synthetic cotton polyploid in upland cotton

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Abstract. Cotton leaf curl disease (CLCuD), caused by a geminivirus complex, is the most serious disease of upland cotton in northwest India and Pakistan. It results in substantial losses in cotton yield and fibre quality. Due to continuous appearance of new viral strains, all the established CLCuD resistant stocks, extant and obsolete cultivars of upland cotton have become susceptible. Therefore, it became crucial to explore the novel sources of CLCuD resistance, as development of CLCuD resistant varieties is the most practical approach to manage this menace. Here, for the first time, we report introgression and mapping of CLCuD resistance from a 'synthetic cotton polyploid' to upland cotton. A backcross population (synthetic polyploid / *Gossypium hirsutum* Acc. PIL 43/*G. hirsutum* Acc. PIL 43) was developed for studying inheritance and mapping of CLCuD resistance. Dominance of CLCuD resistance was observed over its susceptibility. Two dominant genes were found to confer resistance to CLCuD. Molecular analysis through genotyping-by-sequencing revealed that chromosomes A01 and D07 harboured one CLCuD resistance gene each.

Keywords. cotton leaf curl disease; begomoviruses; resistance breeding; genotyping-by-sequencing; *Gossypium*.

Introduction

Cotton is the most important source of natural fibre and is the mainstay of many economies. It is cultivated on an area of 30–36 million hectares in more than 80 countries. Four Asian countries, namely India, China, Pakistan and Uzbekistan account for ~56% of the global cotton production. Of the 28.67 million farmers growing cotton worldwide, 82.1% of them belong to these Asian countries (Kranthi 2019). *Gossypium hirsutum* commonly known as American cotton or upland cotton is the most widely grown cotton species and occupies more than 98% of the cotton acreage worldwide. *G. barbadense*, *G. arboreum* and *G. herbaceum* are the other cultivated cotton species. Cotton production is adversely affected by various biotic and abiotic stresses. Among the biotic constraints, cotton leaf curl disease (CLCuD) is the most serious threat to upland cotton cultivation in northwestern India and Pakistan. This disease has also spread to China (Cai *et al.* 2010). Financial losses due to CLCuD between 1992 and 1997 to Pakistan economy have been reported to be nearly US$ 5 billion (Briddon and Markham 2000). Substantial reduction in seed cotton yield due to CLCuD has been reported in Indian states of Punjab (10.5–92.2%), Haryana (39.4–81.4%) and Rajasthan (32.9–50.3%) (Monga *et al.* 2001). Besides, adverse effects of CLCuD on yield and its component traits, it is also known to deteriorate fibre quality of cotton lint—the major product of cotton (Ahmad *et al.* 2002; Singh *et al.* 2013; Farooq *et al.* 2015; Monga and Sain 2021).

CLCuD is caused by the begomoviruses which belong to the family Geminiviridae. The viral complex consists of a monopartite begomovirus (DNA-A) and single-stranded DNA satellite molecules, namely betasatellite and alphasatellite. Causal complex of the disease is transmitted by an insect vector whitefly (*Bemisia tabaci*). Asia-II is the predominant genetic group of whitefly found in north India

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(Ellango *et al.* 2015; Naveen *et al.* 2017). Association of CLCuD with a geminivirus transmitted by whitefly was first reported by Mansoor *et al.* (1993). However, Briddon *et al.* (2001) unambiguously demonstrated that both begomovirus and DNA β (betasatellite) are required for the successful induction of typical symptoms of CLCuD. The genome of begomovirus consists of a single stranded, circular DNA molecule of about 2.7 kb having seven open-reading frames (ORFs), namely C1, C2, C3, C4 and C5 (in complementary sense) and V1 and V2 (in virion sense). ORFs, namely C1 and C3 are associated with replication, C2 for transcription activation, whereas V1 and V2 participate in packaging. Betasatellite associated with CLCuD is a single-stranded DNA molecule of nearly half (~1.35 kb) the size of begomovirus genome. It encodes a single βC1 protein which acts as pathogenicity determinant (Saeed *et al.* 2005). Replication, encapsidation and movement of betasatellite depends on its helper begomovirus. Alphasatellite (initially known as DNA-1), a single-stranded DNA molecule of about 1.35 kb was found to be associated with CLCuD (Mansoor *et al.* 1999). It is capable of self-replication but depends on begomovirus for insect transmission and for spreading within the plant. Alphasatellite does not play any role in the induction of CLCuD symptoms. Initial symptom of CLCuD is the thickening of small veins on upper young leaves which slowly extend and merge resulting in continuous reticulation of small veins. Other prominent symptom is the upward or downward curling of leaves. In severe cases, there is formation of enation (cup shaped outgrowth) on the lower surface of leaves.

In the Indian subcontinent, CLCuD was first observed near Multan (Pakistan) in 1967 (Hussain and Ali 1975) and this issue prevailed locally in the next couple of decades. Thereafter, the cotton area affected by CLCuD continued to increase from 60 ha in 1988 to 810 ha in 1990, and to 14,000 ha in 1991. The first epidemic of CLCuD occurred in 1992 when the disease was reported in an area of 121,000 ha, which further rose to 202,000 ha in 1993 (Briddon and Markham 2000). Yield losses of 9.05 million bales and 8.04 million bales due to CLCuD have been reported during 1992 and 1993, respectively (Javed *et al.* 2019). Subsequently, the disease spread to the other cotton growing areas of Punjab and other provinces of Pakistan. The next CLCuD epidemic started in Pakistan after the appearance of resistance-breaking Burewala strain during 2001–2002 leading to 100% crop losses in many areas (Rajagopalan *et al.* 2012). In India, CLCuD was reported on upland cotton at Sri Ganganagar, Rajasthan, in 1993 (Ajmera 1994). Due to the movement of vector (whitefly), the disease spread to all the cotton growing areas in northwestern India between 1994 and 1998 (Monga *et al.* 2004). In 1997, CLCuD appeared in epidemic form in Rajasthan and seriously affected cotton production on a sizable area (~100,000 ha) (Monga *et al.* 2011). Cotton cultivation in northwestern India is dominated by transgenic Bt-cotton hybrids which are vulnerable to CLCuD. Reduction in seed cotton yield ranging from 15.7 to 56.7% was registered in All India Coordinated Research Project trials on popular Bt-cotton hybrids at various centres in Punjab, Haryana and Rajasthan, from 2009 to 2014 (Monga 2014).

Development of CLCuD resistant varieties is the most practical approach to manage this menace. In fact, identification of CLCuD resistant donors and development of CLCuD resistant cultivars are the important cotton research activities undertaken at Agricultural Universities in northwestern cotton growing states of India and Pakistan. As a result of breeding efforts, several CLCuD resistant American cotton cultivars, namely LHH 144, CSHH 198, CSHH 238, CSHH 243, F 1861, LH 2076, RS 875, RS 810, RS 2013, H 1117, H 1226 etc. were developed and released for cultivation in northwestern cotton growing states of India (Monga *et al.* 2011). Similarly, in Pakistan, CLCuD resistant American cotton accessions LRA-5166 and CP-15/2 (developed at Central Institute for Cotton Research, Nagpur, India; Chakrabarty *et al.* 2020) were extensively used in breeding programmes for incorporating CLCuD resistance in susceptible cotton varieties (Rahman *et al.* 2017). Several cotton varieties such as CIM-1100, MNH-552, CIM-448, CIM-496, NIBGE-2 and FH-901 resistant to CLCuD were released for cultivation in Pakistan. Notably, NIBGE-2 (developed from an intervarietal cross between LRA-5166 and S-12) was released in 2006 due to its resistance to the most prevalent Multan strain and high tolerance to resistance-breaking Burewala strain of CLCuD (Rahman and Zafar 2007).

However, due to the continuous appearance of new viral strains, all the established CLCuD resistant stocks, extant and obsolete cultivars of upland cotton have become susceptible. Keeping in view the economic importance and narrow gene pool of upland cotton, it became indispensable that novel sources of CLCuD resistance be explored among related cultivated/wild species of cotton. Development of 'synthetic amphiploids' from the progenitor/nonprogenitor species and their hybridization with natural polyploids to create variability is attractive. Generation/use of synthetic amphiploids for the transfer of useful traits in cotton has been reported by several workers (Beasley 1942; Brubaker and Brown 2003; Bell and Robinson 2004; Sacks and Robinson 2009; Zhang *et al.* 2014; Chen *et al.* 2015; Pathak *et al.* 2016). Here, for the first time we report the use of a 'synthetic cotton polyploid' for the introgression and mapping of CLCuD resistance in upland cotton. Genetic analysis and molecular mapping of CLCuD resistance genes will facilitate their precise transfer in the elite cotton varieties and advance lines through marker-aided selection.

Material and methods

Population development

G. hirsutum accession PIL 43 was used as the male parent for the development of initial cross with 'synthetic

polyploid'. A total of 3158 flowers of 'synthetic polyploid' were pollinated. A mixture of growth regulators (GA_3 @ 50 ppm + NAA @ 100 ppm) was applied at the base of pedicle for three consecutive days after pollination to enhance crossed boll retention. Twenty-eight mature crossed bolls were obtained, thus registering a crossed boll retention percentage of 0.88 and 25 F_1 seeds were obtained. Number of seeds per boll ranged from 0 to 2 with an average of 0.89 seed per boll. F_1 hybrids were backcrossed to the recurrent parent PIL 43 to generate BC_1F_1 population. The details on population development are given in Vij *et al.* (2020). Briefly, 1868 BC_1F_1 seeds were obtained after attempting 7434 pollinations. A total of 296 (15.85%) seeds germinated, of which 194 BC_1F_1 plants were established in the field for phenotyping.

Phenotyping of CLCuD

The symptoms of parents, F_1 hybrids and 194 BC_1F_1 plants for susceptibility to CLCuD were visually observed until maturity. Plants showing typical symptoms of the disease (thickening of veins, curling of leaves, presence of enation etc.) (figure 1) were considered CLCuD susceptible, whereas, plants free from disease symptoms were designated as resistant. Row to row and plant to plant spacing was kept 67.5 cm and 60.0 cm, respectively. PIL 43 (CLCuD susceptible recurrent parent) was repeatedly planted in the experimental plot. Besides pots containing susceptible plants of F 846, a highly CLCuD susceptible upland cotton variety, were kept in and around the experimental site so as to supply continuous inoculmn of the disease causing viruses. Whitefly (vector of the CLCuD causing viruses) population was not controlled (by avoiding the use of insecticides) throughout the crop season to ensure the spread of the disease. Number of CLCuD resistant and susceptible plants was counted. Chi-square test was employed for unraveling inheritance of the disease.

Genotyping-by-sequencing (GBS) and data analysis

Genomic DNA was isolated from fresh young leaves collected from parents and individual BC_1F_1 plants following the protocol given by Saghai-Maroof *et al.* (1984). DNA quantity and quality were assessed on 0.8% agarose gel and nano-drop spectrophotometer (Thermo Scientific NanoDrop 8000 Spectrophotometer). DNA samples were outsourced to AgriGenome Labs Private Limited, Hyderabad, India for genotyping using GBS as described by Peterson *et al.* (2012). *Sph*1 and *Mluc*1 enzyme combination was used for preparing GBS library which was sequenced on Illumina HiSeq X platform.

Raw reads obtained after sequencing were filtered by dDocent pipeline (v.2.6.0) using programme Trimmomatic (v.0.38) to remove low quality bases (quality score <20) and adapter sequences. A sliding 5-bp window was applied to trim the bases when the average quality score dropped below 10. Reads were then aligned to the cotton reference genome (https://datadryad.org/stash/dataset/doi:10.5061/dryad.tg557hc) using BWA (v. 0.7.8). SNP calling was conducted through Freebayes software (v.1.2.0) and the resulting bi-allelic SNPs were filtered for read depth $\geq$ 10 using VCFtools. Further, filtering was conducted for INDELs, missing genotypes < 10% and minor allele frequency of 0.05. Thereafter, SNPs monomorphic between parents and showing distorted segregation were removed from further analysis. Final filtered SNP markers along with phenotype were used to map CLCuD resistance using OneMap package (Margarido *et al.* 2007) in RStudio (RStudio Team 2020). Kosambi mapping function was used to estimate the recombination frequency. A logarithm of odds (LOD) value of 3 and maximum recombination fraction of 0.4 was used to estimate map distance between markers. The map distances were drawn using MapChart 2.2 software (Voorrips 2002).

Figure 1. Leaves of (a) CLCuD resistant synthetic polyploid manifesting no disease symptoms. (b) CLCuD susceptible PIL 43 showing vein thickening and enation. (c) and (d) segregants exhibiting leaf curling, vein thickening and enation.

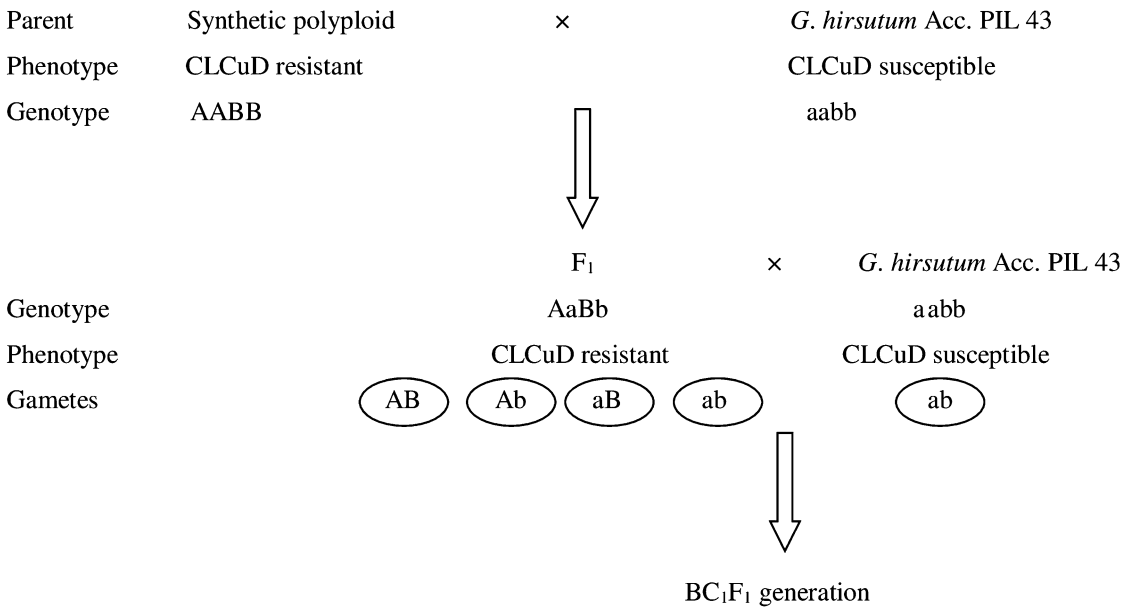
Table 1. Chi-square test of goodness of fit for CLCuD inheritance in BC_1F_1 generation.

Phenotype	Observed number	Expected number	$\chi^2 = (O - E)^2/E$
Resistant	53	48.5	0.42
Susceptible	141	145.5	0.14
Total	194		$\sum = 0.56^{NS}$

^{NS}Nonsignificant differences at 0.05 level of significance.

Results and discussion

Cotton leaf curl disease is the most serious biotic stress and threat to successful cultivation of upland cotton. CLCuD is known to inflict heavy losses in cotton yield and fibre quality. Due to wide host range, availability of large number of cryptic species and invasiveness, management of whitefly is difficult (Vyskocilova *et al.* 2018). Significant resistance of several whitefly populations to many insecticide groups has been reported (Naveen *et al.* 2017). Therefore, management of CLCuD through the control of its vector (whitefly) is practically not feasible. Hence, host plant resistance is the most viable alternative for protecting upland cotton from this devastating disease. Attempts have been made to identify and utilize progenitor and nonprogenitor cotton species for incorporation of CLCuD resistance in upland cotton. Utilizing *G. anomalum*, a wild B-genome cotton species, a high yielding and CLCuD tolerant upland cotton variety CIM-608 has been released in Pakistan during 2013 (Anjum *et al.* 2014). Similarly, another CLCuD tolerant upland cotton variety namely Cyto-124 having *G. anomalum* and *G. arboreum* in its pedigree has been approved for cultivation in Pakistan during 2015. Recently, an upland cotton line, Mac7, resistant to CLCuD has been identified. It has unique pedigree as one of its parents (XG-15) has been developed using a wild *G. hirsutum* accession, whereas the other parent had introgressions from wild *G. raimondii* (Zaidi *et al.* 2020). Although Mac7 is agronomically inferior, it is being used as a donor to transfer CLCuD resistance in upland cotton in India and Pakistan.



Genotype	Phenotype
AaBb	Resistant
Aabb	Susceptible
aaBb	Susceptible
aabb	Susceptible

Phenotypic ratio: 1 (CLCuD resistant) : 3 (CLCuD susceptible)

Figure 2. Flow chart for the development of BC_1F_1 generation and segregation of CLCuD resistance.

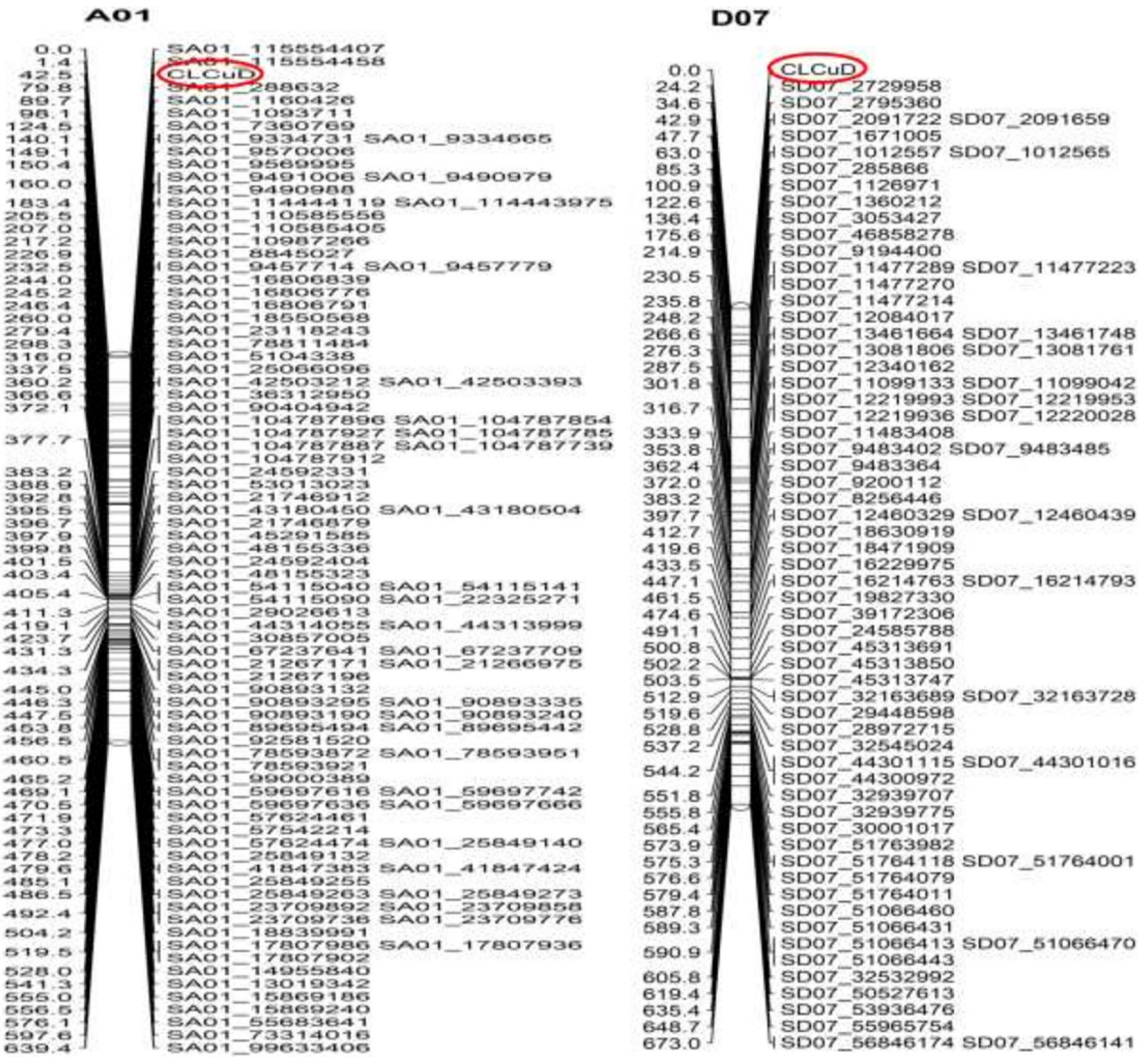


Figure 3. Chromosome maps showing position of mapped genes governing resistance to CLCuD.

Similarly, we identified a CLCuD resistant wild nonprogenitor D-genome cotton species *G. armourianum* (Pathak *et al.* 2016; Suthar *et al.* 2021). Using this species, CLCuD resistance has been introgressed, mapped and CLCuD resistant prebreeding upland cotton lines have been developed (manuscript under preparation).

Inheritance of CLCuD

The prerequisite for successful exploitation of a trait is to determine its inheritance and nature of gene action. The F_1 hybrids derived from synthetic polyploid × *G. hirsutum* accession PIL 43 cross were found to be resistant to CLCuD, suggesting dominant nature of CLCuD resistance over its susceptibility. This observation is consistent with earlier studies of Ali (1997), Aslam *et al.* (2000), Haider *et al.* (2003), Mahmood (2004), Rahman *et al.* (2005), Ahuja *et al.* (2007), Pathak *et al.* (2009), Ahmad *et al.* (2011), Hussain *et al.* (2012), Khan (2013), where dominant expression of CLCuD resistance in upland cotton has been reported. In the present investigation, BC_1F_1 (synthetic polyploid/2**G. hirsutum* Acc. PIL 43) population was used to study the inheritance of CLCuD. Of the 194 BC_1F_1 plants, 53 were found to be CLCuD resistant and rest, 141, were susceptible to the disease (table 1), indicating digenic control of CLCuD resistance. Thus, two dominant genes are required for the manifestation of CLCuD resistant phenotype. Accordingly, genotypes of synthetic polyploid (CLCuD resistant) and PIL 43 (CLCuD susceptible) may be depicted as 'AABB' and 'aabb', respectively for this trait (figure 2). CLCuD resistant and susceptible plants in the BC_1F_1 generation fit in a ratio of 1 (resistant): 3 (susceptible) as revealed by nonsignificant chi-square value of 0.56 at one degree of freedom (table 1). This is in fact, a modification of typical 9:7 ratio obtained for an F_2 population. Thus, it is evident that plants with any of the following genotypes A_bb, aaB_, and aabb would be CLCuD susceptible (figure 2).

Resistance to CLCuD in upland cotton has been reported to be under the control of major genes. For instance,

monogenic inheritance under the control of a single dominant gene has been reported by Ali (1997), Aslam *et al.* (2000), Haider *et al*. (2003), Mahmood (2004), Khan (2013) etc. Two genes with various types of interactions such as duplicate dominant (Iqbal *et al.* 2003; Ahuja *et al.* 2007), duplicate inhibitory (Rahman *et al.* 2005; Ahuja *et al.* 2007) and duplicate recessive (Ahuja *et al.* 2007) have been implicated for CLCuD resistance. Three gene inheritance (triplicate, dominant and epistasis) governing CLCuD resistance has also been reported by Ahuja *et al.* (2007). Similarly, Rahman *et al.* (2005) observed the involvement of three genes (two conferring resistance and one suppressor of resistance) in the inheritance of CLCuD resistance. The differences in genetic control of CLCuD resistance as revealed by the foregoing discussion may be attributed to different parents used in the genetic analysis.

Molecular mapping of genes conferring resistance to CLCuD

Molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple-sequence repeats (SSRs) and single-nucleotide polymorphisms (SNPs) have found various applications in cotton research such as gene/QTL mapping, construction of linkage maps, marker-assisted selection, varietal fingerprinting, germplasm characterization etc. (Pathak *et al.* 2019). SNPs are the most informative molecular markers. GBS refers to detection of SNPs using high-throughput sequencing technologies. It is rapid, specific and highly reproducible technique. It is based on reduced representation sequencing (RRS) and whole genome resequencing (WGR) methods. In the present investigation, genotyping of the parents and individual BC_1F_1 plants was accomplished using GBS technique. Phenotypic data on CLCuD resistance and susceptibility generated on parents and mapping population were associated with genotypic (SNP) data using OneMap package (Margarido *et al.* 2007) in RStudio (RStudio Team 2020). The analysis revealed that chromosomes A01 and D07 harboured one gene each imparting resistance to CLCuD. The gene on chromosome A01 was flanked by markers SA01_115554458 and SA01_288632, which were 41.1 cM and 37.3 cM away from the target gene, respectively. On the chromosome D07, only one marker, SD07_2729958, was found to be associated with the target gene at a distance of 24.8 cM (figure 3).

To the best of our knowledge, this is the first report describing the use of a synthetic cotton polyploid as donor for the introgression and mapping of CLCuD resistance in upland cotton. However, the genes conferring resistance to CLCuD need to be fine mapped so as to facilitate makeraided selection for their precise and efficient transfer to elite varieties and advance lines of upland cotton. Given the vulnerability of cotton cultivars to CLCuD, non-availability of CLCuD resistant donors in upland cotton and cross-compatible Egyptian cotton (*G. barbadense*) and substantial contribution of cotton in India's economy, the availability of CLCuD resistant cotton varieties will go a long way not only for enhancing the production and productivity of cotton but also its sustainability.

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