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GENETIC MONITORING OF INTROGRESSED ALLELES FROM *GOSSYPIUM ARBOREUM* L. INTO *G. HIRSUTUM* L. USING SSR MARKERS: A POTENTIAL APPROACH FOR BRINGING NEW ALLELES UNDER CULTIVATION

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Abstract

In the present investigation, a BC_4F_2 population was developed from a cross [(*2 G. arboreum*) *G. hirsutum*] for transferring resistance to cotton leaf curl disease (CLCuD) from *G. arboreum* into *G. hirsutum*. Both parent species were surveyed with 156 SSR markers, and 19 SSRs were found polymorphic amplifying 81 alleles. Out of these, 31 alleles were informative (nine derived from *G. arboreum* while 24 derived from *G. hirsutum*). Four high yielding BC_4F_2 selected plants were surveyed with these polymorphic SSRs. Two SSR markers (JESPR-156-closely linked with QTLs conferring resistance to CLCuD and MGHES-15-closely linked with genes involved in shaping plant architecture) showed introgression of alleles derived from *G. arboreum* into *G. hirsutum* (BC_4F_2). One novel allele (not derived from either of the parent species) was also amplified in one of the BC_4F_2 plants, possibly generated due to structural changes in cotton genome after polyploidization, or colchicine treatment/ovule culture. These genetic introgressions monitored through DNA markers from alien sources into cultivated gene pool can buffer the potential risks of diseases and the changing environment.

Key words: Introgression, SSRs, Novel allele, *Gossypium*

Introduction

Cotton, a source of natural lint, sustains textile industry worldwide—generates 550 billion US$. Sustainable cotton production is largely challenged by a number of biotic and abiotic factors (Rahman *et al*., 2012; Rahman, 2016). Among these, cotton leaf curl disease (CLCuD) is the most detrimental that suppress cotton production in Pakistan by approximately two million bales annually (Rajagopalan *et al*., 2012; Nazeer *et al*., 2014; Rahman *et al*., 2014). This disease has been spread to other major cotton growing countries like India and China (Cai *et al*., 2010: Singh *et al*., 2013). The disease is also a potential threat for those regions where whitefly is a major or minor pest.

Several control measures were adapted to combat the disease however; development of resistant cotton varieties was found the most effective way to control this disease (Rahman *et al*., 2005; Abbas *et al*., 2015). A number of cotton varieties conferring resistance to this disease were released in Pakistan since the first epidemic observed in early 1990s (Rahman *et al*., 2005; Ahmad *et al*., 2012). This resistance was derived from two cotton genotypes LRA-5166 and CP-15/2, and two resistant and one suppressor genes were involved in conferring resistance to the disease (Rahman *et al*., 2005). This resistance was broken down by the emergence of a new cotton leaf curl virus (CLCV) strain, appeared in Burewala region of Pakistan in 2001 (Briddon, 2003; Mansoor *et al*., 2006; Akhtar *et al*., 2010; Amrao *et al*., 2010). A number of reports are available for developing genetically engineered cotton conferring resistance to CLCuD but none of these has been commercialized (Rahman *et al*., 2016).

Gossypium arboreum L. (till now) is immune and or highly resistant to all naturally occurring viral strains (including Burewala) causing leaf curl disease (Rahman *et al*., 2002, 2005; Iqbal *et al*., 2015). Thus attempts were made to introgress alleles conferring resistance to the disease from *G. arboreum* into *G. hirsutum* using conventional means (Zhang *et al*., 2009; Maqbool *et al*., 2010; Nazeer *et al*., 2014; Rahman *et al*., 2014; Rahman, 2016). However, linkage drags of negatively linked traits ramined a major challenge in introgressing resistance in *G. hirsutm* varieties. Resistance is often lost when selections like adapted parent genotype were made in succeeding backcross populations.

These concerns can be partly addressed using DNA markers for monitoring the introgression of alleles originating from the donor parent (*G. arboreum*) in backcross populations. In recent studies, alleles from *Gossypium australe* and *Gossypium stocksii* were introgressed in *G. hirsutum* (Chen *et al*., 2014; Nazeer *et al*., 2014). In the resultant progenies, fluctuations in expression of the desired traits (resistance to CLCuD) were noticed. DNA markers associated with resistance to the disease are not mapped (using *G. arboreum* as a donor parent). Use of DNA markers can help in selecting plants containing the maximum number of alleles from *G. arboreum* while keeping intact the desirable traits in succeeding backcross populations, thereby accelerating the breeding process (Ulloa *et al*., 2011; Wang *et al*., 2012).

A number of microsatellites markers mapped to chromosomes are available (Yu *et al*., 2011, 2012). These markers can be utilized for monitoring the introgressed parts of genomes derived from *G. arboreum* to minimize losing genomic regions involved in conferring resistance to disease. In this study, we selected markers evenly mapped on all possible chromosomes to ensure maximum genome coverage and reduce sampling errors (Liu *et al*., 2000; Nguyen *et al*., 2004; Guo *et al*., 2007; Yu *et al*., 2012).

Thus objective of the present study was to track the introgressed alleles from *G. arboreum* into selected advanced generations of the [(*2 G. arboreum*) *G. hirsutum*] BC_4F_2 with the aim to introduce the cotton leaf curl disease resistance trait.

Materials and Methods

Developing backcross progenies: The BC_1 progenies were produced in 2011 by backcrossing of F_1 CLCuD-resistant plants (having *G. arboreum* as one of the parent genotype) with *G. hirsutum* and were sown in the cotton field of Cotton Research Station in Multan, Pakistan. Successive backcrossing with *G. hirsutum* (2012-2015) was attemped to raise BC_4F_2 population. The asymptomatic plants showing normal phenotype were selected to perform backcrosses with *G. hirsutum*. The plant progenies showing minor disease symptoms were rejected. The schematic representation of backcross population development is given in Fig. 1.

Screening of backcross population against CLCuD resistance: The resistance /susceptibility to the disease was evaluated using disease scoring scale by examining leaves of each plant, assigned a score using 0 to 4 disease scale (Rahman, unpublished data). While a score '0' assigned to asymptomatic (extremely resistant) plants, '1' for resistant individuals, '2' for tolerant, '3' for susceptible individuals and '4' for highly susceptible plants. Disease scoring data were used to calculate CV (coefficient of variance) with the JMP 5.0 (SAS Institute, Cary, NC, 5.0 PC version) statistical program at 95% confidence level. The yield data and fiber quality traits (GOT, staple length, micronnaire and fiber strength) of all the population were also recorded.

DNA extraction and microsatellite analysis: Parental genotypes of *G. arboreum* species (Mollisoni) and *G. hirsutum* (MNH-886) along with their four selected progenies having higher yield and resistance to CLCuD at BC_4F_2 generation were collected from Cotton Research Station (CRS) Multan and stored at -80°C at the National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan. Genomic DNA was extracted using the CTAB method (Iqbal *et al*., 1997). DNA concentration was measured using Thermo Scientific Nanodrop 2000, USA. Quality of the genomic DNA was tested on 0.8 % agarose gel (Rahman *et al*., 2002).

Molecular analysis was conducted on parental species (*G. arboreum* and *G. hirsutum*) by surveying 156 SSR markers of JESPR, BNL, STV, SHIN, MONCERO, NAU and MGHES series (available in the Plant Genomics and Molecular Breeding lab, NIBGE). These SSR contained 2-10 nucleotide repeat motif. Out of these, 19 primer pairs were found polymorphic and four selected plants of BC_4F_2 generation were screened with these polymorphic SSRs to monitor the introgression of *G. arboreum* alleles. Data of these SSRs including their polymorphic alleles and mapping positions on different chromosomes is given in Table 1.

The PCR amplification was performed in a BIO-RAD C1000 Touch Thermal Cycler. The high fidelity *Taq* DNA polymerase (Thermo Scientific, USA) was utilized for amplifying the genomic DNA in the PCR. Total reaction mixture of 20 µl contained 0.25 mM dNTPs each, 1x Thermo *Taq* buffer, 2.5 mM $MgCl_2$, 0.15 µM primers, one unit *Taq* DNA polymerase (Thermo Scientific USA). Initial denaturation in programming of thermal cycle was carried out for 5 min at 95°C for 1 cycle and 35 cycles each of 94°C for 30 s for denaturation, 50°C primer annealing for 30 s and extension at 72°C for 30 s. The final extension at 72°C was for 10 min. The PCR products were size-fractionated on 6% polyacrylamide gels, visualized via ethidium bromide staining and the gel image was captured using UVP GelDoc-It^2 imager. The PCR reaction was repeated twice to ensure consistency of the amplification.

Scoring of Data and statistical Analysis: *Gossypium arboreum* (var Mollisoni), *G. hirsutum* (MNH-786) and four BC_4F_2 selected plants were investigated using SSR primer pairs. We also calculated the average allele size, and banding profile. The size of the all amplified fragments was estimated by comparing with a 50-bp DNA marker (Thermo Scientific, USA). Null allele was scored for the genotypes which did not amplify a fragment under the given optimized conditions. We also calculated number of amplified alleles in each plant/genotype regardless of the polymorphism. The polymorphic alleles were also counted. The scoring data of all the amplified alleles by polymorphic SSRs is given in Table 1. Following formula was used to calculate the cross-species transferability rate (introgression).

$$\% \text{ of introgression} = \frac{\text{No. of } \textit{G. arboreum} \text{ alleles amplified}}{\text{Total no. of alleles amplified in that progeny}} \times 100$$

BLASTn analysis: Sequence comparison was performed for the introgressing marker MGHES-15 using BLASTn tool with standard search statistics alignment with DNA sequences of all other cotton species to find the homology. It showed 100% homology with *G. hirsutum* (with 2e-92 E value), 98% with *G. arboreum* (6e-87) and 95% with *G. raimondii* (5e-73) vacuole membrane protein KMS1 encoding genes.

Results

Cotton leaf curl virus disease resistance response in BC_4F_2 population: Resistant, moderately tolerant and susceptible plants were recognized based on severity of disease symptoms (Fig. 2). Coefficient of variance (CV) (at a 95% confidence level) showed the presence of significant variation for disease response among the total plants under study. Out of the 500 BC_4F_2 plants, a total of four asymptomatic and high yielding cotton plants were selected to monitor the introgression of *G. arboreum* allele. It is worth mentioning that only the cotton plants having breeding values (agronomically desirable) were selected for genomic analysis. The other plants were rejected. A number of morphological characters were also observed. Few of the plants showed phenotypic resemblance with *G. arboreum* and also with *G. hirsutum* (Fig. 3).

Table 1. Amplified alleles (bp size) by *G. arboreum* var Mollisoni and *G. hirsutum* var MNH-886 parental species and selected four BC_4F_2 progenies by polymorphic primers with their chromosomal mapped position.

S. No.	Marker	Chr. location	*G. arboreum* alleles(bp)	*G. hirsutum* alleles(bp)	BC_4F_2 Progeny-1(bp)	BC_4F_2 Progeny-2(bp)	BC_4F_2 Progeny-3(bp)	BC_4F_2 Progeny-4(bp)
1.	MONCERO-00145	8	0	130	130	130	130	130
			120	120	120	120	120	120
2.	MONCERO-00057	8	0	100	100	100	100	100
			120	0	0	0	0	0
3.	MONCERO-00061	8	155	155	155	155	155	155
			0	130	130	130	130	130
4.	SHIN-940	8	0	190	190	190	190	190
			195	0	0	0	0	0
5.	JESPR-156	14	110	0	110	0	0	0
			0	85	0	85	85	85
			0	80	80	80	80	80
6.	BNL-3280	18/20	210	210	210	210	210	210
			0	0	0	0	215	0
			0	260	260	260	260	260
7.	JESPR-304	2	210	0	0	0	0	0
			0	200	200	200	200	200
			0	190	190	190	190	190
8.	JESPR-131	8	135	0	0	0	0	0
			0	150	150	150	150	150
			0	155	155	0	155	155
9.	BNL-2634	16	0	200	200	200	200	200
			250	250	250	250	250	250
10.	BNL-834	17	125	125	125	125	125	125
			0	150	150	150	150	150
11.	BNL-116	26	140	0	0	0	0	0
			0	150	150	150	150	150
			0	160	160	160	160	160
12.	BNL-448	22	100	100	100	100	100	100
			190	190	190	190	190	190
			205	205	205	205	205	205
			0	210	210	210	210	210
13.	BNL-3255	8	0	250	250	250	250	250
			300	300	300	300	300	300
			340	0	0	0	0	0
			0	360	360	360	360	360
			400	400	400	400	400	400
14.	BNL-4095	15	200	200	200	200	200	200
			0	210	210	210	210	210
15.	BNL-2967	12	150	150	150	150	150	150
			0	160	160	160	160	160
16.	STV-80	13	0	245	245	245	245	245
			250	250	250	250	250	250
17.	MONCERO-0105	8	145	145	145	145	145	145
			0	155	155	155	155	155
			170	0	0	0	0	0
18.	MGHES-15	5	200	0	200	0	0	0
			0	190	190	190	190	190
			0	180	180	180	180	180
19.	NAU-5467	14	0	240	240	240	240	240
			200	200	200	200	200	200

Common alleles amplified in both genotypes= 15; Informative alleles (polymorphic in either of the genotype)=34; Novel allele (not present in either of the genotypes)=1; Introgression in progeny-1= 5.26%

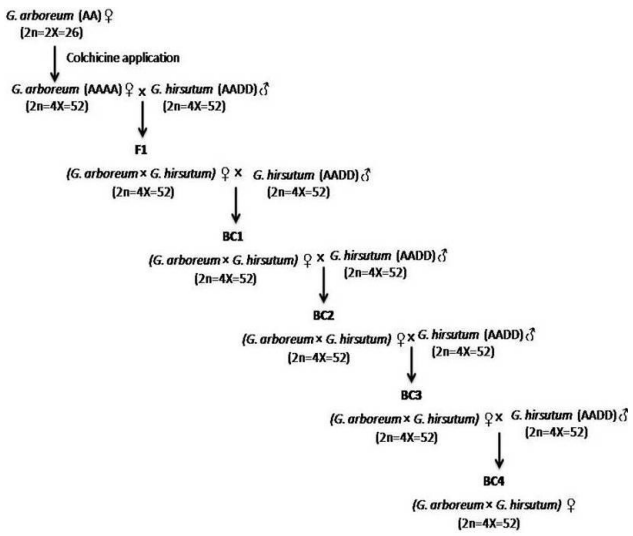

Fig. 1. Schematic representation of pedigree development for backcross population of *G. arboreum* var Mollisoni and *G. hirsutum* var MNH-886.

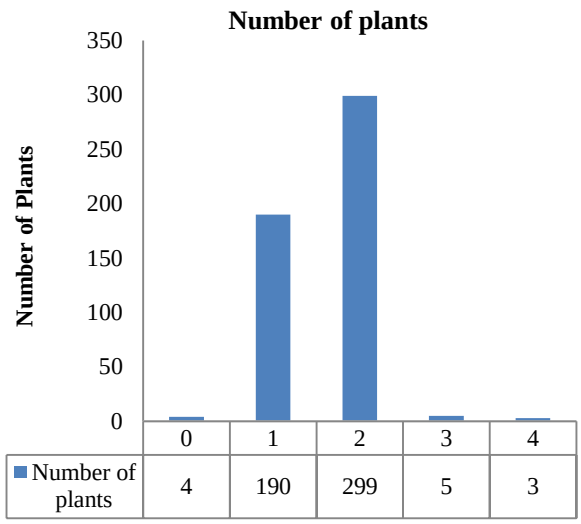

Fig. 2. Frequency of Cotton leaf curl disease scores in the BC_4F_2 population showing a normal distribution of phenotypic variation for the trait.

Fig. 3. Comparative pictures of *G. arboreum* var Mollisoni and *G. hirsutum* var MNH-886 with four selected BC_4F_2 progenies.

Microsatellite analysis for introgression: The genomic DNA of parent genotypes, i.e. *G. arboreum* var Mollisoni and *G. hirsutum* var MNH-886 along with their four progenies at BC_4F_2 generation were genotyped using publicly available SSR markers representing different series including JESPR, BNL, SHIN, SSRs, NAU and EST-SSR such as MGHES and STV. Out of the 156 SSRs (amplified 191 alleles), 19 were found polymorphic, amplified 81 alleles. Out of these, nine alleles were amplified in *G. arboreum* which were used for tracking the introgression of *G. arboreum* alleles into the four selected BC_4F_2 progenies. Two alleles amplified by JESPR-156 and MGHES-15 in *G. arboreum* were only detected in one of the BC_4F_2 progenies (Fig. 4a and Fig. 4b). Amplification of these two alleles in this particular plant suggested that introgression rate from *G. arboreum* to *G. hirsutum* is ~5.2 %. It is worth mentioning that this plant was found asymptomatic to the disease. The marker JESPR-156 was mapped on chromosome # 14 of *G. hirsutum* (Yu *et al*., 2012) in close proximity of disease resistance QTLs and marker MGHES-15 showed 100% homology with vacuole membrane protein KMS1 encoding genes by BLAST n analysis (Fig. 5).

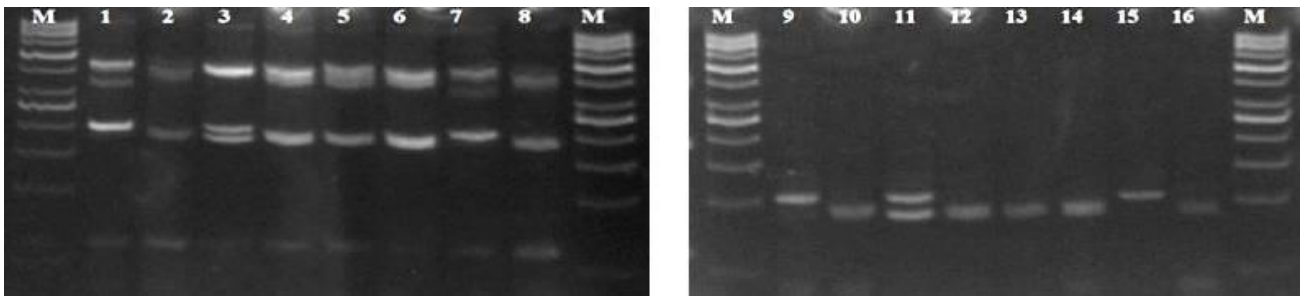

Fig. 4a. Amplification profile of *G. arboreum* var Mollisoni and *G. hirsutum* var MNH-886 parental genotypes and four BC_4F_2 progenies by introgressing markers MGHES-15. Lane M = 50 bp DNA ladder, Lane 1, 7 = *G. arboreum* var Mollisoni, Lane 2, 8= *G. hirsutum* var MNH-886, Lane 3= BC_4F_2 progeny-1, Lane 4= BC_4F_2 progeny-2, Lane 5= BC_4F_2 progeny-3, Lane 6= BC_4F_2 progeny-4. Lane 3= introgression of 200 bp allele from *G. arboreum* var Mollisoni in BC_4F_2progeny-1.

Fig. 4b. Amplification profile of *G. arboreum* var Mollisoni and *G. hirsutum* var MNH-886 parental genotypes and four BC_4F_2 progenies by introgressing markers JESPR-156. Lane M = 50 bp DNA ladder, Lane 9, 15 = *G. arboreum* var Mollisoni, Lane 10, 16= *G. hirsutum* var MNH-886, Lane 11= BC_4F_2 progeny-1, Lane 12= BC_4F_2 progeny-2, Lane 13= BC_4F_2 progeny-3, Lane 14= BC_4F_2 progeny-4. Lane 11= introgression of 110 bp allele from *G. arboreum* var Mollisoni in same BC_4F_2 progeny-1.

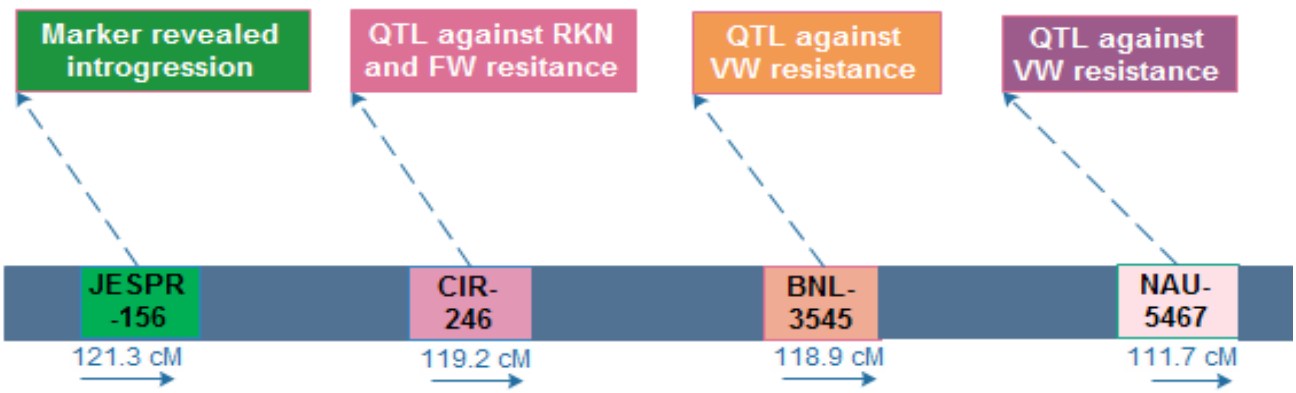

Fig. 5. Marker JESPER-156 (revealed introgression) and nearby markers reported as QTLs against various pathogens; RKN= Root knot nematode; FW= Fusarium wilt; VW= Verticillium wilt

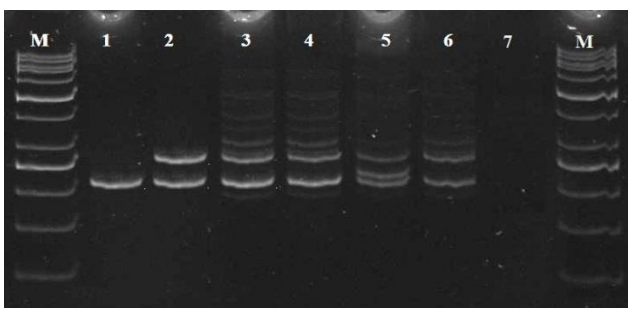

Fig. 6. Amplification of a novel allele by marker BNL-3280. Lane M = 50 bp DNA ladder, Lane 1= *G. arboreum* var Mollisoni, Lane 2= *G. hirsutum* var MNH-886, Lane 3= BC_4F_2 progeny-1, Lane 4= BC_4F_2 progeny-2, Lane 5= BC_4F_2 progeny-3, Lane 6= BC_4F_2 progeny-4. Lane 5= novel allele of 215 bp in BC_4F_2 progeny-3, not amplified by any of the parental genotypes, Lane 6= negative control without DNA template.

In cotton, availability of DNA markers (functional or structural) with high allelic variability which are transferable to other cotton species is important for undertaking genetic analysis. For example such markers can help in conducting QTL mapping, comparative mapping, gene-flow and allelic profile studies in cotton (Tabbasam *et al*., 2014). In this study, each of the alleles amplified by JESPR-156 and MGHES-15 in *G. arboreum* was introgressed in one of the BC_4F_2 plants showing the low introgression rate of donor genome in succeeding backcross populations. An interesting finding of the present investigation is the identification of a novel allele (215 bp long, not amplified in both the parental genotypes) by marker BNL-3280 in one of the selected BC_4F_2 plants (Fig. 6).

Discussion

The genetic diversity of cultivated tetraploid cotton (*G. hirsutum*) against CLCuD can be widened by exploiting G. *arboreum* as a donor parent, a well-known resistant source against various diseases (Wang *et al*., 2012; Nazeer *et al*., 2014). However, due to genome incompatibilities introgression of *G. arboreum* disease resistant genes into *G. hirsutum* and in their successive advanced backcross populations require strict monitoring which is quite difficult by conventional means. Here we report SSR markers revealing introgression of *G. arboreum* alleles into advanced backcross BC_4F_2 populations. Most of the markers used in this study were mapped on different chromosomes (Yu *et al*., 2011; Yu *et al*., 2012). The advantage of surveying with the already mapped markers is that the over representation of certain genomic regions can be

avoided. The rate of introgression of *G. arboreum* alleles in BC_4F_2 progenies is low primarily due to crossing of the recurrent genotype (MNH-886) several times. Secondly, plants in each generation were selected like the recurrent parent for attempting the next backcross. This practice eliminates or reduces the genomic regions introgressed from the donor parent.

It was found that several markers associated with resistance to different diseases including root knot nematodes, Fusarium wilt and verticillium wilt and have been reported in flanking region of JESPR-156 marker that revealed introgression of *G. arboreum* in BC_4F_2 progeny. The marker JESPR-156 introgressed successfully in one of the asymptomatic plants was mapped on chromosome # 14 of *G. hirsutum* (Yu *et al*., 2012) which was 9.6 cM distantly apart from the marker NAU-5467 (associated with QTL conferring resistance to Verticilum wilt) (Wang *et al*., 2014, Zhang *et al*., 2015) and found non segregating in this population. Thus this region needs to be explored at length for cloning disease resistant genes. The second marker (derived from EST) was not mapped on chromosomes of either of the cotton species. After doing sequence comparisons, this marker was mapped on chromosome #5 of *G. raimondii*. The BLASTn alignment of this sequence showed a significant homology with genes involved in encoding vacuole membrane protein KMS1 of *Gossypium.* In most plant species, these proteins have conserved sequence and are possibly involved in plant secretary pathway, maintenance of ER integrity and in the organization or functioning of ER/Golgi interface for plant cell architecture, vacuolar trafficking thus central to endomembrane fusion (Wang *et al*., 2011). In a recent study, QTLs associated with improved fiber traits in this region were identified (Iqbal & Rahman 2017). Introgressed alleles in one of the four selected plants make it the best target for advancing the generation leading towards the development of new cotton variety and or for future breeding purposes.

Since MGHES-15 has been derived from EST-SSR, and this locus is evolutionary conserved which may have role in development of cotton plant architecture. It is worth mentioning that all these markers have been derived from EST-SSRs and SSRs of *G. hirsutum*, and are successfully amplified in both the cotton species. Transferability between different species has also been demonstrated in several crop species (Kuleung *et al*., 2004; Saha *et al*., 2004) and also in the genus *Gossypium* (Tabbasam *et al*., 2014).

An interesting finding of the present investigation was the amplification of novel allele in the advanced backcross population by marker BNL-3280 (linked with fiber elongation QTLs, Shen *et al*., 2005). There can be several possible explanations for amplification of this allele. Cross pollination (5-10% in Pakistan dominantly done by honey bee) may increase the likelihood of contamination by the other genotypes growing nearby cotton field (Rahman & Zafar 2010). However, in the present study, controlled pollination was carried out-hence chances of contamination by cross pollination were negligible. Secondly, cotton genome may undergo probable structural changes due to transposition or translocations after genome doubling (by colchicine) and interspecific hybridization which may generate new alleles in the progenies. Such commonalities have been reported in other crops including Brassica and synthetic wheat where quick but dynamic fluctuations in genome structure, including non-additive transferability of genomic regions and genome-specific sequence deletion were reported (Liu *et al*., 1998, Chen & Ni 2006). Contrary to this, tolerance to these genomic changes after genome doubling and interspecific hybridization was reported in cotton (Liu *et al*., 2001). In another report, newly resynthesized allopolyploid *Brassica napus* maintained at least one locus methylated (in parental state) while modification in the second locus resulted in the evolution of a novel allele (Lukens *et al*., 2004, 2006). This interpretation is consistent with highly repetitive genome of cotton (Paterson *et al*., 2012; Li *et al*., 2015). Thus it is suggested that evolution of a novel allele during the process of resynthesizing of allotetraploid cotton is possible like many other crops in advanced BC_4F_2 generations. Further studies are needed to establish this fact in cotton.

Conclusion

It is concluded that there is possibility of transferring CLCuD resistant genes from *G. arboreum* to *G. hirsutum*. Moreover, DNA markers are useful in monitoring the introgression of alleles/genomic regions originating from the wild species into the cultivated species—thus concern of linkage drag and high genetic similarity can overcome. Also, resynthesizing allotetraploid cotton by hybridizing two ancestral species may produce novel alleles which can enhance the genetic diversity. The informative SSR markers identified in the present study will be useful for initiating marker-assisted breeding in future—a step towards sustaining fiber security.

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