Development of an Interspecific Hybrid (Triploid) by Crossing *Gossypium hirsutum* and *G. arboreum*

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Received April 12, 2010; accepted March 28, 2011

Summary The 2 species *G. hirsutum* and *G. arboreum* were crossed and triploid hybrid (AAD) was developed as a first step of crossing. The triploid is sterile and its hybridity was confirmed with morphological traits and DNA based SSR markers. The chromosome analysis at metaphase-I exhibited 13AA bivalents and 13D univalents at most of the PMC's studied which also confirmed its triploid nature. The mean chromosome pairing association was recorded as 12.90% (univalent), 10.45% (bivalent), 0.45% (trivalent), and 0.48% (tetraivalent). The formation of multivalents depicted genome association between 2 species. The further utility of synthesized triploid with respect to the development of CLCuV resistant introgression genotypes is discussed

Key words Triploid, *G. hirsutum*, *G. arboreum*, Interspecific hybrid, Cotton leaf curl virus (CLCuV), *In vitro*.

Cotton is believed to be life line of the national economy of the country. During the last crop season, cotton staked in agriculture with 7.4% and 22.7% in value addition of major crops, grew modestly by 1.4% and its production, at 11.8 million bales, is below par (GOP, 2008–09). Cotton cultivation is mainly confined to Punjab and Sindh provinces with a few pockets in Baluchistan in relatively hot and dry environments. Cotton production in the Punjab province of Pakistan is mainly under threat due to attack of insect pests and cotton leaf curl virus disease. Among the insects, sucking pests *viz* whitefly (*Bamisia tabicii*), jassids (*Amrasca bigutulla*) and thrips (*Thrips tabaci* Lind) are posing the most serious threats and their population has tremendously increased in the last 4 to 5 years. It has been estimated that sucking pests alone are responsible for a 25–30% loss in lint and seed yield (Parnell *et al.* 1949). The cotton leaf curl virus disease is also playing havoc in the cotton belt of Punjab and its incidence is endemic in Sindh province also.

All commercial varieties having earlier resistant genes CP15/2, LRA 5166, Cedix and Kivi-1021 (UI-Islam and Iqbal 2002) are in the grip of the cotton leaf curl virus, mainly due to a narrow genetic base of genotypes which are succulent and hence attract the sucking pests and bring disease into effect through its vector whitefly. Therefore, resistance/tolerance to cotton leaf curl virus is mandatory nowadays in every cotton breeding program. Concerted efforts are being made to defy virus attacks by screening the subsequent generations of *G. hirsutum* crosses at hotspots of disease and eventually selecting high yielding disease tolerant genotypes. The success to have outstanding tolerance against disease is very low. The difficulty in generating a resistant genotype is posed by the mutant versions and new recombinants of CLCV virus (Mansoor *et al.* 2003). Hence, strategies to harbor new disease resistant genes from diploid wild species of cotton (*G. arboreum*, *G. ananolum*) to cultivated upland cotton (*G. hirsutum*) are being launched to develop cultivars that possess built-in resistance to sucking insects and cotton leaf curl virus. The diploid species *G. arboreum*, *G. herbacium* and *G. ananolum* are completely immune to cotton leaf curl virus.

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Therefore, the introgression of useful characteristics should be an important method (Liang *et al.* 2002, Saravanan *et al.* 2007). However, transfer of cotton leaf curl virus resistance from *G. arboreum* to *G. hirsutum* is a tedious process because of cross incompatibility, hybrid breakdown and hybrid sterility related to ploidy differences. Earlier studies revealed that introgression improves the productivity, fibre properties (Tayyab 1990, Liang *et al.* 2002, Mergeai 2003) and resistance to pests and diseases (Lukefahr *et al.* 1971, Avila *et al.* 2005). The present investigation reports a triploid with genetic constitution. (AAD) as a first step in successful hybridization between *G. arboreum* and *G. hirsutum*. The new hybrid is totally self-sterile and its interspecific status was confirmed using SSR markers and chromosome analysis. Among the available DNA fingerprinting methods SSR technique is most reliable and easy to perform. The scientists have consensus that SSR is the best technique for hybrid identification in cotton (Asif *et al.* 2009) and rice (Ye-Yun *et al.* 2005).

Materials and methods

Plant material

An accession *G. hirsutum* C.V. S.A-6654 and *G. arboreum* C.V. FDH-228 were selected for interspecific hybridization from gene pool of Cotton Research Institute, Faisalabad, Pakistan and grown in field during crop season 2007–08 with standard agronomic practices. The crossing was made effected by using following scheme.

1. Male Parent: (*G. arboreum* 2n. 2x=26)
2. Female Parent: (*G. hirsutum* 2n. 2x=52)

Crossing procedure and ovule culture

The appropriate flower buds were hand emasculated during late evening and pollinated in the next morning. The mixture of growth regulators at the concentration of 50 mg l⁻¹ NAA and 100 mg l⁻¹ GA were applied after pollination at the base of pedicle for 20 days to reduce boll shedding and have ovules with good developed endosperms. The bolls were collected, surface sterilized and ovules were cultured *in vitro* on modified MS medium as described by (Stewart and Hsu 1977, 1978). The seedlings derived from cultured embryos were raised in small pots containing compost under controlled conditions of the lab facility. After proper temperature conditioning, seedlings were transferred in earthen pots at greenhouses for their growth and development in a natural environment.

Morphological characters

Data was collected on morphological characters *viz* growth habit, stem color, leaf color, leaf incision, leaf shape, leaf texture, leaf veins, petiol color, leaf hairiness, leaf nectricness, stem pubescence, position of stigma, corolla color, anther color, stigma length, petal spot, petal length, petal width, bracket length, bracket width and anther number of parents and a triploid hybrid and compared to describe the hybridity of triploid.

DNA based confirmation of hybridity

Young leaves from a triploid and parents were collected early in the morning. The leaves were transported to the laboratory in ice and stored at –20°C. The standard CTAB method was used for DNA extraction (Doyle and Doyle 1990).

DNA concentration in the samples was measured using Nanodrop Spectrophotometer (ND-1000, Nanodrop, USA) at wave lengths of 260 and 280 nm. Quality of DNA was observed by running 50 ng DNA on 0.8% agarose gel. The quantity of DNA was also confirmed by comparing with Quantification Standards Phage λ DNA (Fermentas) on 0.8% agarose gel. All DNA samples

were diluted to a working concentration of 15 ng/ μ l and stored at -20°C in 50 μ l aliquots. The heterozygosity of triploid was determined by using SSR primers from BNL series (<http://www.cottonmarker.org/projects/bnl/index.shtml>). After literature search, 10 primers reported to be polymorphic in previous studies were selected for hybridity testing in the present study. Two SSR primers (Table 1)

showed polymorphism and co-dominance clearly differentiating the parents and hybrid. The SSR primers were amplified in Eppendorf thermocycler (Mastercycler gradient, 96 wells) by following protocol used by Ullah (2009). The amplicons were resolved through electrophoresis using 3% high resolution agarose gel for 1 h 20 min at 100 V. The amplicons were visualized under UV after staining with ethidium bromide using G Box, Syngene Gel documentation system. The first and eighth wells of the gel were loaded with 50 bp DNA ladder (Gene RulerTM, #SM0373, Fermentas, USA) for determination of exact size of amplicons. Second, third and fourth well of the gel were *G. arboreum*, *G. hirsutum* and interspecific hybrid (triploid) respectively. The parents and triploid were repeated in the fifth, sixth and seventh wells of the gel with second primer.

Table 1. Polymorphic SSR primers and their sequences

SSR-primers	Sequence
BNL-1317	F. AAAAAATCAGCCAAATTGGGA R. CGTCAACAATTGTCCCAAGA
BNL-2634	F. AACAAACATTGAAAGTCGGGG R. CCCAGCTGCTTATTGGTTTC

Chromosome studies

The young flower buds of appropriate stages of both parents and a triploid were collected from a single plant between 7.30 to 8.30 a.m. and fixed in 95% ethanol : glacial acetic acid (3 : 1) for 48 h and then preserved in 70% ethanol until use. Cytological smears of pollen mother cells (PMCs) were squashed in 1% acetocarmine on microscopic slides. After squashing of anthers, slides were slightly warmed to get good spread of chromosomes. The appropriate meiotic stages were photographed with Olympus research microscope.

Results

Crossability and ovule culture

A few triploids have been obtained by first accomplishing hybridization and then culturing the ovules *in vitro* to maturity. The survival of triploid hybrids till maturity was poor which indicated the effect of zygotic barriers. The seed setting between *G. hirsutum* and *G. arboreum* crosses, ovules cultured and number of viable hybrids obtained are depicted in Table 2. Out of 210 cultured ovules only 5 could be established in the field.

Morphological characters

The triploid plant resembled the parental parent in habit. The expression of morphological characters of parents and triploid hybrid is depicted in Table 3. The growth habit, stem color, leaf incision, leaf shape, position of stigma, anther color and petal spot resembled the parental parent. However, corolla color expression was intermediate of both parents. The petal spot expression of parental parent was of light color and intensity in triploid (Fig. 1A–C). The expression of leaf shape in triploid hybrid is vigorous (Fig. 1D) The size of stigma height, petal length, petal width, bracket length and anther numbers in triploid were higher than both of parents (Table 3).

DNA based confirmation of hybridity

Out of 40 SSR primers used in the study, 4 SSR primers were found to be polymorphic between the 2 parents, and out of these, only 2 primers differentiated the hybrid from parents. The amplification profile of these primers showed that these are co-dominant. The results of extremely

Table 2. Crossability, boll setting and embryo development in interspecific hybridization

Cross	No. of flowers crossed	No. of bolls with large embryos	No. of ovules cultured	No. of triploids obtained	No. of triploids survived till maturity
<i>G. hirsutum</i> × <i>G. arboreum</i>	80	39	210	12	5

Table 3. Morphological characters of parents and hybrid (triploid)

Sr. No	Characters	<i>G. hirsutum</i>	<i>G. arboreum</i>	<i>G. hirsutum</i> × <i>G. arboreum</i>
1	Habit	Annual	Annual/intermediate	Perennial/intermediate
2	Stem color	Green	Light reddish	Light reddish
3	Leaf color	Green	Green	Green
4	Leaf incision	Shallow	Deep	Deep
5	Leaf shape	Palmate	Okra	Okra
6	Leaf texture	Medium smooth	Medium smooth	Medium smooth
7	Leaf veins	Thick prominent	Thick prominent	Thick prominent
8	Petiol color	Green	Green	Green
9	Leaf hairiness	Slight hairy	Absent	Slight hairy
10	Leaf nectarines	Present	Present	Present
11	Stem pubescence	Slight hairy	Slight hairy	Slight hairy
12	Position of stigma	Protruded	Embedded	Embedded
13	Corola color	Cream	Yellow	Creamy with yellow shade at base
14	Anther color	Cream	Yellow	Yellow
15	Stigma height	1.5 cm	1.4 cm	1.8 cm
16	Petal spot	Absent	Dark red	Light pink
17	Petal length	2.7 cm	2.5 cm	3.8 cm
18	Petal width	3.1 cm	2.4 cm	4.1 cm
19	Bracket length	1.5 cm	1.5 cm	2.5 cm
20	Bracket width	1.0 cm	1.6 cm	1.0 cm
21	Anther number	58	65	75

polymorphic SSR primers BNL-1317 and BNL-2634 are depicted in Fig. 1F. These results clearly showed that sample given in the fourth and seventh lane is hybrid of parents (*G. arboreum* and *G. hirsutum*). As with primer BNL-1317, first parent amplified DNA fragment of 185 bp compared to the DNA fragment of 200 bp which was amplified by the second. This primer amplified different size fragments in the parents from the same loci of the genome showing the presence of different alleles of the same gene in 2 parents. This was confirmed from the amplification profile of the hybrid with primer BNL-1317 which showed the presence of both alleles in the hybrid. This loci depicted co-dominance and hybridity status of the offspring was confirmed from these results. Both of these primers individually can be used in hybridity confirmation. The results of the present study not only confirmed the hybridity status but also proved that SSR technique may successfully be used in such cases.

Cytogenetics studies

The meiosis in both *G. arboreum* (AA) and *G. hirsutum* (AADD) was regular and normal and both species showed 13 and 26 bivalents at Metaphase-I respectively (data not presented here). Theoretically, 13AA bivalents and 13D univalents are expected at metaphase-I in triploid hybrid. Therefore, expected configuration (13II+13I) was maximum and observed in 34.48% of MPC's, thus confirmed triploid hybrid (Fig. 1E and Table 4). Univalent, bivalent, trivalent and quadrivalent chromosome associations were also recorded at various frequencies among 29 PMC's as depicted in Table 4. In the triploid under study, the number of univalents and bivalents ranged

Fig. 1. Interspecific hybridization between *G. hirsutum* and *G. arboreum*

A) Flower of *G. hirsutum*, B) flower of *G. arboreum*, C) flower of triploid hybrid, D) left to right leaves of *G. arboreum*, triploid hybrid and *G. hirsutum*, E) 39 chromosomes (26A, 13D) in triploid hybrid. F) gel picture showing DNA amplification profile of parents and hybrid with polymorphic SSR primers BNL-1317 and BNL-2634 (Left to Right) M-50bp DNA ladder, P1-*G. arboreum*, P2-*G. hirsutum*, Hyb-triploid hybrid)

from 12 to 14 and 8 to 13 with an average of 12.90 and 10.45 respectively. The number of trivalents and quadvalents ranged from 0 to 1 with an average of 0.45 and 0.48 respectively.

Discussion

The present investigation was primarily carried out to synthesize a triploid (AAD) as a first step of hybridization between 2 species (*G. hirsutum* × *G. arboreum*). We report success in the

Table 4. Cytological observation of chromosome association in triploid hybrid of *G. hirsutum* × *G. arboreum*

PMC studied	Type and No. of associations per PMCs				% PMCS
	I (Univalent)	II (Bivalent)	III (Trivalent)	IV (Tetrapivalent)	
10	13	13	—	—	34.48
8	12	10	1	1	27.58
6	13	8	—	1	20.68
5	14	9	1	—	17.24
Total=29	374	303	13	14	—
Mean	12.90	10.45	0.45	0.48	—

development of such a triploid hybrid. This triploid is sterile due to ploidy barriers and variation of the chromosomes. Upland cotton (*Gossypium hirsutum* L.) is reproductively isolated from *G. arboreum* L. via post-zygotic breeding barriers (Sacks 2008). Therefore, in further studies, polyploidisation of triploid (AAD) will be carried out for its fertility restoration (Stewart 1995). The normal hybridization between diploid and tetraploid species is hindered due to differences in ploidy level and biological barriers, therefore, photohormones *viz* GA3 and NAA were sprayed in combination after pollination to enhance fertilization and metabolism in generative organs of maternal plant (Liang *et al.* 2002). The results showed that number of ovules with good developmental endosperm were cultured to maturity. The fertilized ovules were of bigger size and composition of medium described by Stewart and Hsu (1977) for seedling development worked perfectly and permitted embryos for active root and shoot formation. Our investigations demonstrated that 5.7% of cultured ovules developed into seedlings. The ovules with degenerated and less vigorous embryos did not germinate. The haploid nature of triploid was established with morphological, cytological and DNA based data. The synthesized triploid is intermediate in nature. The partial dominance type of expression of corolla color and petal spot make it distinct from its parents, which is in accordance with the observations of Sacks (2008). Moreover, stem color, leaf incision, leaf shape, position of stigma, anther color, of *G. arboreum* were dominant as the F1 triploid (AAD) exhibited these characters. The transgressive effect for stigma height, petal length, petal width, bracket length and anther numbers is also observed in triploid. The parents and triploid were also subjected to cotton leaf curl virus stress by growing them near hotspots of the disease. Since whitefly is a vector for disease, therefore its infestation was not controlled chemically during crop season. The *G. hirsutum* completely succumbed to disease and absolutely no symptoms of CLCuV appeared on *G. arboreum* and triploid. From this, it is assumed that CLCuV resistance of *G. arboreum* can be transferred to *G. hirsutum* by carefully developing disease resistant introgression lines. The strategy will be based on the restoration of the fertility status of the triploid by chromosome doubling and repeated backcrossing of hexaploid with disease susceptible, quality fibre and high yielding *G. hirsutum* cultivar. The cytogenetic determination of subsequent tetraploid segregants and DNA based marker assisted selection of CLCuV resistant genes would be beneficial in the development of disease resistant introgressed genotypes. The DNA based data is also reliable for identification of interspecific hybrids because the hybrids between tetraploid and diploid is distinct from its parents for chromosome number and pairing. Utilized SSR markers support the hybridity of triploid, which is in accordance with earlier reports of Mehetre *et al.* (2004) and Saravanan (2007). The inheritance of SSR markers of both parents was depicted in hybrids which explains the presence of parts of parental genomes in the triploid hence confirming the success of hybridization among 2 species.

The cytogenetics analysis depicted 39 chromosomes (26A, 13D) in triploid. The main purpose of cytogenetic analysis was to confirm the hybrid nature of triploid which is fairly achieved in the present investigation. Some of the PMCs were further studied to have a look on pairing behavior of

chromosomes in triploid with genetic constitution AAD. The mean pairing association 12.90% (univalent), 10.45% (bivalent), 0.45% (trivalent), and 0.48% (tetraivalent) was depicted in our data which did not differ much from earlier report of Vafaie-Tabar and Chandrashekaram (2007). The presence of trivalents and quadrivalents indicated pairing affinity exists between genomes involved. Since, both genomes (A and D) of triploid possess different number and size, therefore, homeologous chromosomes rarely pair due to different size. The 13 chromosome observed in this study belongs to D genome and their univalents behavior can be attributed due to asynapsis or desynapsis. The difference in degree of chromosome condensation can be the main force controlling regular pairing behavior in *Gossypium* (Endrizzi 1962).

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