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Molecular confirmation of *Gossypium hirsutum* chromosome substitution lines

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Abstract The primary gene pool for tetraploid cotton species includes *Gossypium hirsutum* L., as well as the other four 2n = 52 species of *Gossypium* (*G. barbadense*, *G. mustellinum*, *G. tomentosum* and *G. darwinii*). To help overcome barriers to effective introgression, we have developed a number of alien chromosome substitution (CS) lines from *G. barbadense*, *G. mustellinum* and *G. tomentosum*, most of which are nearly isogenic to the inbred 'Texas Marker-1', a genetic standard. At the time CS line development was initiated, molecular markers did not exist for some CS lines, and most of these lines were developed based on cytological analysis without using any marker-based testing. Here we report on tests with SSR markers from one ore more linkage maps specific to the substituted chromosome or chromosome segment from one or more linkage maps to assess the constitution and genetic identity of the CS lines. The specific objective of this paper is to report on the genetic identity of the CS lines using SSR markers and some special characteristics associated with some of the CS lines. We used chromosome-specific SSR markers following standard methods of DNA extraction, PCR and according to manufacturer's protocol on ABI Genetic Analyzer 3130xl to confirm the identity of the introgressed alien chromosome or chromosome segments in the CS lines. For most CS lines and most mapped markers, the observed SSR profiles were concordant with expectations as per the results of cytological analysis. For a minority of markers and lines, however, the results were discordant; these markers, linkage groups, and CS lines will be further investigated to understand and define their genetic identity for use as breeding resources. Interspecific germplasm introgression can be useful for genetic

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improvement of Upland cotton. However, such efforts are constrained by genetic incompatibilities between the species. Our results document for the first time the development of CS lines from *G. tomentosum* and *G. mustelinum*. These CS lines will open a new paradigm in cotton breeding program by providing a tool for introgression of useful genes from wild and unadapted species in the genetic improvement of Upland cotton.

Keywords Cotton · Chromosome substitution lines · SSR markers

Introduction

The narrow genetic base of Upland cotton cultivars, *Gossypium hirsutum* L. was one of the major constrains contributing to the recent declines in fiber yield and quality (Esbroeck and Bowman 1998; Paterson et al. 2004). Wild species and unadapted types are useful genetic resources that can be tapped to broaden the germplasm base. The primary gene pool for cotton consists of the natural tetraploid (AD genome, 2n = 52) species including *G. hirsutum* L. $(AD)_1$, *Gossypium barbadense* L. $(AD)_2$, *Gossypium tomentosum* Nutt. ex Seem $(AD)_3$, *Gossypium mustelinum* Watt $(AD)_4$ and *Gossypium darwinii* Watt $(AD)_5$. These species have 26-chromosome haploid genomes with grossly similar A–D genomic architecture and produced fertile hybrids. These characteristics justify selection of these species to be the first line of choice to tap valuable exotic gene pools to broaden the germplasm base of Upland cotton lines. However, the exotic gene pools of wild species and unadapted germplasms in *Gossypium* spp. have been under-characterized and under-utilized in the genetic improvement of Upland cotton because of technological and biological challenges associated with conventional methods of interspecific introgression. Interspecific chromosome substitution lines in which a pair of chromosomes or chromosome segments has been substituted with a pair of alien species' chromosomes or chromosome segments are useful tools that complement conventional methods of interspecific introgression (Saha et al. 2006, 2011; Bernacchi et al. 1998; Eshed and Zamir 1994).

About 10 years ago, we developed 17 backcrossed chromosomal substitutions (CS-B) line (Stelly et al. 2005). Through hypoaneuploid-based backcross chromosome substitution, pairs of chromosomes (or chromosome arms) in TM-1 (*G. hirsutum*) were replaced by the respective homozygous chromosome (or chromosome arm) pair from *G. barbadense* line 3–79. The methods of each CS-B line development were discussed in our previous studies (Stelly et al. 2005; Saha et al. 2011). This method included the following stages: (i) create isogenic Upland chromosome-deficient stocks, by backcrossing various chromosome deficiencies (monosome or monotelodisome) to a common line, namely 'Texas Marker-1' (TM-1); (ii) cross the TM-1 hypoaneuploid with the donor to create a F_1 substitution stock that is monosomic or monotelodisomic (i.e., partially hemizygous), then backcross the F_1 and succeeding $BCnF_1$ hypoaneuploid to isogenic cytogenetic stock as a recurrent seed parent; (iii) inbreed the BC_5F_1 hypoaneuploid derivative to recover a euploid disomic substitution line.

We also documented that chromosome substitution lines are useful tools to introgress novel genes from other species in Upland cotton (Saha et al. 2006, 2011, 2013). In addition to the released CS-B lines, we extended our study recently with chromosome substitution line development for *G. tomentosum* and *G. mustelinum* and some other chromosomes of *G. barbadense*. These substitution lines are nearly isogenic to the common parent TM-1 for 25 chromosome pairs, as well as to each other, for 24 chromosome pairs. These lines were developed based on diagnostic metaphase I meiotic configurations in microsporocytes (Stelly et al. 2005; Saha et al. 2011).

However, we could not confirm the genetic identity of all of the CS lines based on chromosome-specific molecular markers of these CS lines because very few chromosome-specific molecular markers were available at that time in the public domain. Recently the genetic identity of several of the released CS-B lines have been confirmed by molecular markers (Buyyarapu et al. 2011). The overall objective of this paper is to report on: (1) some novel phenotypes associated with the chromosome substitution lines from *G. tomentosum* and *G. mustelinum* and some other chromosomes of *G. barbadense* and (2) confirmation on the cytogenetic identity of the CS lines using chromosome-specific SSR markers.

Materials

The interspecific chromosome substitution lines for this study were developed at Texas A&M University,

primarily by cytogenetically modified backcross-inbred breeding. Each line was developed using a specific hypoaneuploid type of *G. hirsutum*, e.g., primary monosomic for chromosome 1 (H01) or acrocentric ("monotelodisomic") for the long arm of chromosome 8 (Te08Lo) or tertiary monosomic for parts of chromosomes 10 and 19 (NTN10-19), as the recurrent parent for hybridization and backcrossing until the BC_5F_1 hypoaneuploid F_1 was recovered. In most cases, the recurrent hypoaneuploid parent had been previously backcrossed to inbred TM-1 and was thus quasi-isogenic to it. Upon identification, a hypoaneuploid BC_5F_1 segregate was selfed to identify a disomic, true-breeding disomic substitution. The substitution lines are named according to the expectedly substituted alien chromosome or segment(s), e.g., CS-B01, CS-M08sh or CS-T10-19, where B, M and T refer to the species of origin, *G. barbadense*, *G. mustelinum* or *G. tomentosum*, respectively. We followed the nomenclature procedure for the CS lines as per our previous report (Stelly et al. 2005).

The chromosome substitution lines were grown in fields at the Plant Science Research Farm at Mississippi State, MS for increase and field evaluation of all the lines. Standard agronomic technologies and irrigation systems were used. We recorded the morphology of the CS lines in two successive years in four replicated plots at two different locations. Leaf samples from CS lines were collected for DNA analysis.

Methods

DNA extraction

From each chromosome substitution line and its parents, two or three young, fully expanded leaves were collected, lyophilized at −40 °C and genomic DNA were isolated from approximately 10 mg of the leaf tissues, using DNAeasy cotton genomic DNA extraction protocol (Qiagen kit) which was optimized for robotic extraction. Extracted genomic DNAs were checked with Nanodrop, and diluted to appropriate concentrations before using for PCR.

PCR

PCR-amplifications were performed in a 10 µl reaction mix containing 1 µl 10× PCR buffer, 0.2 µl dNTPs (10 mM each), 1.2 µl 25 mM $MgCl_2$, 0.3 µl 5 µM labeled primers (FAM, HEX, VIC, PET), 0.1 µl AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), and 5 ng/µl genomic DNA. PCR amplification was carried out using a PTC-225 DNA Engine Tetrad thermocycler (MJ Research, USA) with first denaturation at 95 °C for 4 min, followed by 10 cycles of 94 °C for 1 min, 55 °C for 30 s (decreases of 0.5 °C in each cycle) and 72 °C for 1 min; 39 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30:00 min. A final 6 min extension at 72 °C was performed.

ABI

With labelled primers, polymorphisms among chromosome substitution lines at amplified SSR loci were visualized in a denaturing capillary electrophoresis according to manufacturer's protocol on Genetic Analyzer 3130xl (Applied Biosystems, Foster City, CA, USA). PCR products were diluted 1:20 before loading into capillaries. The size standards of LIZ 500 or ROX 400 were loaded with the diluted PCR-products according to the manufacturer's guidelines (Applied Biosystems, USA). Calling the size of amplified products was performed using Gene Mapper 4.0 (Applied Biosystems, USA) as well.

Results and discussion

The development of chromosome substitution lines in cotton by cytogenetically modified backcross-inbreeding is facilitated by differential transmission of hypoaneuploidy via *G. hirsutum* mega- versus micro-gametophytes. Though maternal rates vary widely among the specific chromosomal deficiencies, each hypoaneuploid condition is typically transmissible via mega-gametophytes but not micro-gametophytes (Endrizzi et al. 1985; Stelly et al. 2005; Saha et al. 2011). Thus, cytogenetically selected hypoaneuploid segregates will not only remain non-recombinant for the hemizygous chromosome (or segment), when used as a pollen parent, they will typically transmit that intact alien chromosome (segment) to all progeny.

The cytogenetic behavior, gamete transmission and patterns of inheritance for CS lines are similar to TM-1, the recurrent parent, except that each line differs by the replacement of a specific homologous pair of chromosomes or chromosome segments from the donor alien species (Stelly et al. 2005). TM-1, an

inbred line, was developed by Kohel et al. (1970) from the commercial cultivar Deltapine 14 and has been maintained over 50 generations by self-pollination (Kohel et al. 1970). Line 3–79, originated as a doubled-haploid (Endrizzi et al. 1985), used as the donor parent for the substituted chromosome or chromosome segments from *G. barbadense* and labeled as CS-B lines. A plant of *G. tomentosum* and another plant of *G. mustelinum*, maintained at the Beasley Laboratory, Texas A&M University, was used as the donor parent for the substituted chromosome or chromosome segment and labeled as CS-T and CS-M, respectively, for the CS lines. The overall method of the CS line development was discussed in our previous reports (Stelly et al. 2005; Saha et al. 2011). A euploid BC_5S_1 26II plant was selected on the basis of phenotype and cytological analysis of the metaphase-I microsporocytes at the last step of CS line development, and selfed to establish the corresponding euploid BC_5S_1 CS line.

The initial development of a euploid BC_5S_1 ($2n = 52$) plant for a CS line was based on plant phenotypes and cytological analysis of the metaphase-I microsporocytes. The cytological observation that euploid F_1 and BC_nF_1 hybrids typically form 26 ring bivalents (IIs) at metaphase I, compared to the diagnostic metaphase-I meiotic configurations of monosomic and monotelodisomic hybrids that include a univalent (I) or an unequal rod bivalent (Ii), 25 II + I or 25 II + Ii, respectively (Stelly et al. 2005). While, the conventional cotton karyotyping techniques suffice in many ways, they are limited by small chromosome size, low heterochromatic polymorphism and minimal karyotypic differences between AD species genomes (Gardunia 2006). A number of interspecific F_1 hypoaneuploids have been assessed with SSRs (Liu et al. 2000; Gutierrez et al. 2009), but this is the first systematic characterization of advanced chromosome substitution lines using currently available chromosome-specific SSR markers (Table 1).

Molecular marker analysis

For molecular analysis on the genetic identity of disomic chromosome substitution intermediates, we followed the principles of deletion molecular analysis, such as loss of heterozygosity (Liu et al. 2000; Gutierrez et al. 2009) (Fig. 1). Due to absence of the *G. hirsutum* chromosome (segment), the chromosome substitution line CS line is expected to carry only the allele of the donor alien species specific to the substituted alien chromosome or chromosome segment and will lack the respective TM-1 allele, the recurrent parent, allele specific to the locus of the substituted chromosome or chromosome segment (Saha et al. 2011). The detailed molecular results (Table 1) suggest that the observed SSR profiles were concordant for most of the chromosome substitution lines. For example, in CS-B05sh line the short arm of chromosome five of TM-1, *G. hirsutum*, was substituted with the short arm of chromosome five from *G. tomentosum*. Our molecular results from deletion analysis showed the presence of only the *G. tomentosum* allele for the short arm of chromosome five in CS-T05sh line and missing the TM-1 allele (Fig. 1). We also confirmed the recovery of the recurrent parental alleles for the non-substituted chromosomes by running several markers specific to chromosomes other than the substituted chromosome. Our results were concordant with the cytological diagnostic results of the CS lines showing the presence of only TM-1 alleles for the other chromosomes or chromosome segments and alien species allele for the substituted chromosome or chromosome segment. Many of the results were concordant with and thus substantiated by previous molecular maps of SSR markers including BNL2921 associated with lint yield on chromosome one, BNL3408 linked to lint percentage on chromosome three, BNL3241 associated with micronaire, CIR 328 linked with seed cotton yield and JESPR65 associated with fiber wall thickness and maturity on the short arm of chromosome five, BNL3650 associated with lint percentage on chromosome six, CIR148 associated with fiber span length on chromosome 12, CIR216 associated with maturity on chromosome 18 (Wu et al. 2009), and BNL1395 associated with lint percentage on chromosome seven, CIR307 linked to fiber strength on the short arm of chromosome 15, BNL827 associated with fiber strength on chromosome 25 (Qin et al. 2015).

However, for a minority of markers and lines, the results were discordant with expectations. The lack of congruence suggests that these markers, linkage groups, and CS lines should be investigated further.

Morphological characteristics

All of the CS lines produced fertile flowers and seeds with both fuzz and lint fibers with overall morphology

Table 1 Confirmation of cytological results with chromosome specific SSR markers

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
BNL2921	154	158	NA	NA	158	CS-B01	Confirmed for chromosome 01
BNL3580	212	207	NA	NA	207	CS-B01	Confirmed for chromosome 01
BNL3848	227	248	NA	NA	248	CS-B01	Confirmed for chromosome 01
BNL3888	184	197	NA	NA	197	CS-B01	Confirmed for chromosome 01
CIR009	222, 228	233	NA	NA	222, 233	CS-B01	Confirmed for chromosome 01
NAU2437	231, 239	235	NA	NA	235, 239	CS-B01	Confirmed for chromosome 01
BNL1693	241	NA	240, 244	NA	244	CS-T01	Confirmed for chromosome 01
BNL2921	154	NA	162	NA	162	CS-T01	Confirmed for chromosome 01
BNL3580	212	NA	202	NA	202	CS-T01	Confirmed for chromosome 01
BNL3848	227	NA	223	NA	223	CS-T01	Confirmed for chromosome 01
BNL3888	184	NA	182	NA	182	CS-T01	Confirmed for chromosome 01
NAU2437	231, 239	NA	247	NA	239, 247	CS-T01	Confirmed for chromosome 01
BNL1434	248	264	NA	NA	264	CS-B02	Confirmed for chromosome 02
JESPR101	127	120	NA	NA	120	CS-B02	Confirmed for chromosome 02
JESPR179	174	152	NA	NA	152, 174	CS-B02	Confirmed for chromosome 02
JESPR304	143	121	NA	NA	121	CS-B02	Confirmed for chromosome 02
BNL3972	242	NA	NA	229	229	CS-M02	Confirmed for chromosome 02
DPL0674	231	NA	NA	229	229	CS-M02	Confirmed for chromosome 02
JESPR179	174	NA	NA	211, 214	211, 214	CS-M02	Confirmed for chromosome 02
BNL3972	242	NA	229	NA	229	CS-T02	Confirmed for chromosome 02
NAU2277	131, 136	NA	110	136	110	CS-T02	Confirmed for chromosome 02
BNL1379	267, 340	276, 295	NA	NA	278, 295	CS-B03	Confirmed for chromosome 03
BNL226	225, 229	213, 233	NA	NA	213, 225	CS-B03	Confirmed for chromosome 03
BNL3398	93, 195	94, 121, 167	NA	NA	94, 125, 167, 178	CS-B03	Confirmed for chromosome 03
BNL3408	117, 130, 144	117, 134, 172	NA	NA	117, 134, 144	CS-B03	Confirmed for chromosome 03
NAU862	106	167, 180, 199	NA	NA	167, 180, 192	CS-B03	Confirmed for chromosome 03
BNL2821	192	180, 208	NA	NA	180, 192	CS-B04	Confirmed for chromosome 04
BNL3089	128	124	NA	NA	124	CS-B04	Confirmed for chromosome 04
BNL3988	138, 141	120	NA	NA	120	CS-B04	Confirmed for chromosome 04
CIR222	274, 291	276	NA	NA	276, 291	CS-B04	Confirmed for chromosome 04

Table 1 continued

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
CIR249	192	190	NA	NA	190	CS-B04	Confirmed for chromosome 04
BNL2821	192	180, 208	NA	NA	180, 192	CS-B_NTN04*-15	Confirmed for chromosome 04
BNL3988	139, 141	120	NA	NA	120	CS-B_NTN04*-15	Confirmed for chromosome 04
CIR222	274, 291	276	NA	NA	276, 291	CS-B_NTN04*-15	Confirmed for chromosome 04
Gh107	246, 358	NA	NA	351	351	CS-M04	Confirmed for chromosome 04
Gh107	246, 358	NA	NA	351	351	CS-M_NTN04*-15	Confirmed for chromosome 04
Gh107	246, 358	NA	361	NA	361	CS-T_NTN04*-15	Confirmed for chromosome 04
BNL2732	175, 193, 209	NA	175, 195, 196	NA	175, 194, 196	CS-T05sh	Confirmed for chromosome 05
BNL3241	120	NA	124	NA	124	CS-T05sh	Confirmed for chromosome 05
BNL3995	195	NA	177	NA	177	CS-T05sh	Confirmed for chromosome 05
CIR328	199, 216	NA	199, 209	NA	199, 209	CS-T05sh	Confirmed for chromosome 05
JESPR65	92, 106, 113, 136	NA	97, 106, 124	NA	92, 106, 113, 124	CS-T05sh	Confirmed for chromosome 05
BNL3359	208	NA	NA	217	217	CS-M06	Confirmed for chromosome 06
CIR203	259	NA	NA	239	239	CS-M06	Confirmed for chromosome 06
Gh433	116, 175, 178	NA	NA	106, 202	116, 202	CS-M06	Confirmed for chromosome 06
TMB0154	260	NA	NA	240	240	CS-M06	Confirmed for chromosome 06
CIR203	259	NA	245	NA	245	CS-T06	Confirmed for chromosome 06
Gh433	116, 175, 178	NA	111, 137	NA	137	CS-T06	Confirmed for chromosome 06
TMB0154	260	NA	246	NA	246	CS-T06	Confirmed for chromosome 06
BNL1064	141	NA	158, 159	NA	158, 159	CS-T06	Confirmed for chromosome 06
BNL2569	73, 158, 166	NA	73, 160, 172	NA	73, 158, 172	CS-T06	Confirmed for chromosome 06
BNL2884	152, 162, 163	NA	162, 173	NA	162, 173	CS-T06	Confirmed for chromosome 06
BNL3650	355	NA	340	NA	340	CS-T06	Confirmed for chromosome 06
BNL3987	194, 199, 213	NA	194, 199, 207, 220	NA	194, 199, 207, 220	CS-T06	Confirmed for chromosome 06
BNL1395	153, 161	NA	145, 163	NA	153, 163	CS-T07	Confirmed for chromosome 07
BNL1597	212	NA	228	NA	228	CS-T07	Confirmed for chromosome 07
CIR141	194	NA	170	NA	170	CS-T07	Confirmed for chromosome 07
CIR169	137	NA	135	NA	135	CS-T07	Confirmed for chromosome 07
Gh548	110	NA	110, 113	NA	110, 113	CS-T07	Confirmed for chromosome 07
BNL1017	123, 139	123, 133	NA	NA	123, 133	CS-B08sh	Confirmed for chromosome 08
BNL2772	180	190	NA	NA	190	CS-B08sh	Confirmed for chromosome 08

Table 1 continued

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
BNL3792	232	213	NA	NA	213	CS-B08sh	Confirmed for chromosome 08
BNL3556	135	130	NA	NA	130	CS-B08sh	Confirmed for chromosome 08
JESPR232	154	142	NA	NA	142	CS-B08sh	Confirmed for chromosome 08
BNL3556	135	NA	NA	130	130	CS-M08sh	Confirmed for chromosome 08
BNL3627a	183	NA	NA	165, 185	185	CS-M08sh	Confirmed for chromosome 08
CIR343	223, 237	NA	NA	229, 236	229, 237	CS-M08sh	Confirmed for chromosome 08
JESPR232	154	NA	NA	132, 163	132, 163	CS-M08sh	Confirmed for chromosome 08
BNL1162	242	234	NA	NA	234	CS-B09	Confirmed for chromosome 09
BNL1414	136	125	NA	NA	125	CS-B09	Confirmed for chromosome 09
BNL2590	182, 188	204	NA	NA	182, 204	CS-B09	Confirmed for chromosome 09
BNL0256	206	187	NA	NA	187	CS-B10	Confirmed for chromosome 10
BNL1160	365, 371	374	NA	NA	365, 374	CS-B10	Confirmed for chromosome 10
BNL2960	149	183	NA	NA	183	CS-B10	Confirmed for chromosome 10
BNL3563	227	195, 210	NA	NA	195, 210	CS-B10	Confirmed for chromosome 10
CIR400	154	156	NA	NA	156	CS-B10	Confirmed for chromosome 10
Gh058	169	93	NA	NA	93	CS-B10	Confirmed for chromosome 10
Gh199	75	109, 111	NA	NA	75, 111	CS-B10	Confirmed for chromosome 10
BNL1160	365, 371	373	NA	NA	364, 373	CS-B_NTN10*-19	Confirmed for chromosome 10
BNL3563	227	195, 210	NA	NA	195, 210	CS-B_NTN10*-19	Confirmed for chromosome 10
Gh058	169	NA	160	NA	160	CS-T10	Confirmed for chromosome 10
Gh058	169	NA	160	NA	160	CS-T_NTN10*-19	Confirmed for chromosome 10
BNL3442	111, 131	111, 144	NA	NA	111, 144	CS-B11sh	Confirmed for chromosome 11
BNL3592	201	195	NA	NA	195	CS-B11sh	Confirmed for chromosome 11
BNL625	241	232	NA	NA	232	CS-B11sh	Confirmed for chromosome 11
BNL1066	133	148	NA	NA	148	CS-B_NTN17-11*	Confirmed for chromosome 11
BNL3592	200	194	NA	NA	194	CS-B_NTN17-11*	Confirmed for chromosome 11
BNL625	241	231	NA	NA	231, 241	CS-B_NTN17-11*	Confirmed for chromosome 11
BNL3442	130	NA	NA	113, 152	110, 152	CS-M11sh	Confirmed for chromosome 11
BNL3442	130	NA	110, 133	NA	110, 133	CS-T11sh	Confirmed for chromosome 11
BNL1034	219, 221	NA	213, 250	NA	219, 250	CS-T11sh	Confirmed for chromosome 11
BNL1404	215, 230	NA	215, 222	NA	214, 222	CS-T11sh	Confirmed for chromosome 11

Table 1 continued

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
BNL1681	107, 122	NA	110, 112	NA	112, 122	CS-T11sh	Confirmed for chromosome 11
BNL3411	197	NA	200, 215	NA	200, 215	CS-T11sh	Confirmed for chromosome 11
BNL4094	168, 179, 180	NA	188, 189	NA	188, 189	CS-T11sh	Confirmed for chromosome 11
BNL1673	197	188	NA	NA	188	CS-B12	Confirmed for chromosome 12
CIR148	148	137	NA	NA	137	CS-B12	Confirmed for chromosome 12
BNL2709	136	119	NA	NA	119	CS-B_NTN12*-19	Confirmed for chromosome 12
BNL3835	157, 172, 191	157, 172, 186	NA	NA	157, 172, 186	CS-B_NTN12*-19	Confirmed for chromosome 12
BNL391	283, 307	287, 301, 356	NA	NA	283, 287, 301	CS-B_NTN12*-19	Confirmed for chromosome 12
CIR042	85, 244	89, 249	NA	NA	87, 248	CS-B_NTN12*-19	Confirmed for chromosome 12
CIR148	147	136	NA	NA	136	CS-B_NTN12*-19	Confirmed for chromosome 12
BNL2882	202	211	NA	NA	210	CS-B14sh	Confirmed for chromosome 14
BNL3545	108, 116, 139, 178	107, 114, 129, 216	NA	NA	107, 114, 129, 177	CS-B14sh	Confirmed for chromosome 14
BNL3644	184, 190	187	NA	NA	187	CS-B14sh	Confirmed for chromosome 14
CIR246	167	156	NA	NA	156	CS-B14sh	Confirmed for chromosome 14
BNL3502	154	NA	149, 152	NA	152	CS-T14sh	Confirmed for chromosome 14
BNL3644	184, 190	NA	170, 183	NA	170, 183	CS-T14sh	Confirmed for chromosome 14
CIR181	149	NA	152	NA	152	CS-T14sh	Confirmed for chromosome 14
CIR246	167	NA	152	NA	152	CS-T14sh	Confirmed for chromosome 14
BNL1666	121, 141	121, 132	NA	NA	121, 132	CS-B_NTN04-15*	Confirmed for chromosome 15
BNL3902	179, 193	159, 172	NA	NA	159, 172	CS-B_NTN04-15*	Confirmed for chromosome 15
BNL4082	169	160, 161	NA	NA	160, 161	CS-B_NTN04-15*	Confirmed for chromosome 15
CIR307	177	NA	NA	166	166	CS-M15sh	Confirmed for chromosome 15
TMB1910	193, 204	NA	NA	209	209	CS-M15sh	Confirmed for chromosome 15
BNL1667	162	NA	170	NA	170	CS-T_NTN04-15*	Confirmed for chromosome 15
BNL1395	153, 161	153, 157	NA	NA	157, 163	CS-B16	Confirmed for chromosome 16
BNL3008	129	138	NA	NA	138	CS-B16	Confirmed for chromosome 16
BNL3065	190	182	NA	NA	182	CS-B16	Confirmed for chromosome 16
CIR175	98	94	NA	NA	94	CS-B16	Confirmed for chromosome 16
BNL3065	189	181	NA	NA	181	CS-B_NTN16*-15	Confirmed for chromosome 16
BNL3500	61, 81	63, 111	NA	NA	63, 111	CS-B_NTN16*-15	Confirmed for chromosome 16
TMB2068	No band	123, 133	NA	NA	124, 133	CS-B_NTN16*-15	Confirmed for chromosome 16

Table 1 continued

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
Gh002	92	NA	NA	85	85	CS-M_NTN16*-15	Confirmed for chromosome 16
Gh002	92	NA	75	NA	75	CS-T_NTN16*-15	Confirmed for chromosome 16
BNL1606	191	187	NA	NA	187	CS-B17	Confirmed for chromosome 17
BNL2443	127, 153	129, 167	NA	NA	127, 167	CS-B17	Confirmed for chromosome 17
BNL2471	196	191	NA	NA	191	CS-B17	Confirmed for chromosome 17
BNL2496	98, 113	98, 130	NA	NA	98, 130	CS-B17	Confirmed for chromosome 17
BNL3371	167, 195	167, 191	NA	NA	167, 191	CS-B17	Confirmed for chromosome 17
CIR251	188	192	NA	NA	192	CS-B17	Confirmed for chromosome 17
TMB2018	235	258	NA	NA	258	CS-B17	Confirmed for chromosome 17
BNL2443	127, 153	129, 167	NA	NA	127, 167	CS-B_NTN17*-11	Confirmed for chromosome 17
BNL2471	196	191	NA	NA	191	CS-B_NTN17*-11	Confirmed for chromosome 17
BNL3371	168, 195	168, 191	NA	NA	168, 191	CS-B_NTN17*-11	Confirmed for chromosome 17
BNL1606	191	NA	189	NA	189	CS-T17	Confirmed for chromosome 17
BNL2496	98, 113	NA	98, 121	NA	98, 121	CS-T17	Confirmed for chromosome 17
TMB2018	235	NA	241	NA	241	CS-T17	Confirmed for chromosome 17
BNL0193	109, 116	109, 111	NA	NA	109, 111	CS-B18	Confirmed for chromosome 18
BNL3479	243	251	NA	NA	251	CS-B18	Confirmed for chromosome 18
CIR216	137	141	NA	NA	141	CS-B18	Confirmed for chromosome 18
Gh501	149, 198	146, 201	NA	NA	149, 201	CS-B18	Confirmed for chromosome 18
TMB2762	201	205	NA	NA	201, 205	CS-B18	Confirmed for chromosome 18
BNL0193	109, 116	NA	109, 113	NA	109, 113	CS-T18	Confirmed for chromosome 18
BNL1721	190	NA	160, 171	NA	160, 171	CS-T18	Confirmed for chromosome 18
BNL243	103, 129	NA	114, 135	NA	103, 135	CS-T18	Confirmed for chromosome 18
BNL3479	243	NA	244	NA	244	CS-T18	Confirmed for chromosome 18
BNL569	136, 143	NA	139, 142	NA	142	CS-T18	Confirmed for chromosome 18
CIR216	138	NA	141	NA	141	CS-T18	Confirmed for chromosome 18
CIR216	137	NA	141	NA	141	CS-T18	Confirmed for chromosome 18
BNL1671	105	106, 125	NA	NA	106, 125	CS-B_NTN10-19*	Confirmed for chromosome 19
BNL2821	192	180, 207	NA	NA	166, 207	CS-B_NTN10-19*	Confirmed for chromosome 19
BNL1671	105	106, 125	NA	NA	106, 125	CS-B_NTN12-19*	Confirmed for chromosome 19
BNL2821	192	180, 207	NA	NA	166, 207	CS-B_NTN12-19*	Confirmed for chromosome 19

Table 1 continued

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
BNL358	126	130	NA	NA	130	CS-B22Lo	Confirmed for chromosome 22
BNL3873	124	141	NA	NA	141	CS-B22Lo	Confirmed for chromosome 22
BNL4049	153	149, 159	NA	NA	159	CS-B22Lo	Confirmed for chromosome 22
CIR218	163, 174	165, 174	NA	NA	165, 174	CS-B22Lo	Confirmed for chromosome 22
Gh330	106	98	NA	NA	98	CS-B22Lo	Confirmed for chromosome 22
BNL2609	122, 124, 135, 148	121, 124, 135, 166	NA	NA	121, 124, 135, 166	CS-B22sh	Confirmed for chromosome 22
BNL4015	117	103, 121	NA	NA	121	CS-B22sh	Confirmed for chromosome 22
BNL4030	102, 110	103, 112, 117	NA	NA	108, 117	CS-B22sh	Confirmed for chromosome 22
BNL4030	110	113, 117	NA	NA	117	CS-B22sh	Confirmed for chromosome 22
BNL4092	204, 208	204, 217	NA	NA	204, 217	CS-B22sh	Confirmed for chromosome 22
BNL448	202, 213	200, 202	NA	NA	200, 202	CS-B22sh	Confirmed for chromosome 22
Gh330	106	98	NA	NA	98, 106	CS-B22sh	Confirmed for chromosome 22
CIR218	163, 174	NA	NA	167, 18	165, 174	CS-M22sh	Confirmed for chromosome 22
Gh330	106	NA	NA	103	103	CS-M22sh	Confirmed for chromosome 22
BNL4030	102, 110	NA	112, 121	NA	121	CS-T22sh	Confirmed for chromosome 22
BNL448	202, 213	NA	201, 202	NA	201, 202	CS-T22sh	Confirmed for chromosome 22
BNL150	122	126, 128	NA	NA	122, 126	CS-B25	Confirmed for chromosome 25
BNL2691	216, 239	216, 232	NA	NA	216, 232, 239	CS-B25	Confirmed for chromosome 25
BNL3190	165	159	NA	NA	159, 165	CS-B25	Confirmed for chromosome 25
BNL3436	177, 196	178, 192	NA	NA	178, 192, 196	CS-B25	Confirmed for chromosome 25
BNL3806	175, 210	175, 183	NA	NA	175, 183, 210	CS-B25	Confirmed for chromosome 25
BNL827	265	257	NA	NA	257, 265	CS-B25	Confirmed for chromosome 25
CIR407	145, 155	164, 176	NA	NA	155, 176	CS-B25	Confirmed for chromosome 25
BNL2495	195	185	NA	NA	185	CS-B26Lo	Confirmed for chromosome 26
BNL3510	133	149	NA	NA	149	CS-B26Lo	Confirmed for chromosome 26
BNL3816	202	185	NA	NA	185	CS-B26Lo	Confirmed for chromosome 26
BNL840	158	149	NA	NA	149	CS-B26Lo	Confirmed for chromosome 26
BNL2557	224	224, 233	NA	NA	224, 233	CS-B26Lo	Confirmed for chromosome 26
BNL3599	193	178, 212	NA	NA	212	CS-B26Lo	Confirmed for chromosome 26
BNL2495	182, 195	172, 185	NA	NA	172, 185	CS-B26Lo_New	Confirmed for chromosome 26
BNL2725	107, 110	84	NA	NA	84	CS-B26Lo_New	Confirmed for chromosome 26

Table 1 continued

SSR primer	Base pair size of the SSR marker						Confirmation of alien chromosome (ch) based on both molecular and cytological analysis
	G. hirsutum (*TM*-1)	*G. barbadense* (3–79)	*G. tomentosum*	*G. mustelinum*	CS line	CS line name	
BNL3510	133	149	NA	NA	149	CS-B26Lo_New	Confirmed for chromosome 26
BNL3816	160, 167, 188, 202	162, 172, 186	NA	NA	160, 167, 171, 185	CS-B26Lo_New	Confirmed for chromosome 26
BNL840	157	149	NA	NA	148	CS-B26Lo_New	Confirmed for chromosome 26
BNL2557	224	224, 233	NA	NA	224, 233	CS-B26Lo_New	Confirmed for chromosome 26
BNL3599	193	178, 212	NA	NA	193, 212	CS-B26Lo_New	Confirmed for chromosome 26
BNL1045	213	NA	202, 213	NA	202, 213	CS-T26Lo	Confirmed for chromosome 26
BNL2557	224	NA	224, 239	NA	224, 239	CS-T26Lo	Confirmed for chromosome 26

CS-B indicated chromosome substitution line of *G. barbadense* and the number indicated specific chromosome number, CS-T indicated chromosome substitution lines of *G. tomentosum* and CS-M designated chromosome substitution line of *G. mustelinum*. The numbers in NTN line suggested it contain two homozygous segment of the specific alien chromosomes. NA indicated not relevant with reference to the donor parent for the substituted chromosomes or chromosome segments

like TM-1, the recurrent Upland cotton parent (Fig. 2). Most of the CS lines did not have phenotypes like their donor parents (Fig. 3). Some CS lines were phenotypically distinctive from TM-1 (Table 2; Fig. 4), suggesting the phenotype could have been inherited through the substituted alien chromosome or chromosome segment (Fig. 4). For example, CS-B18 produced "open-bud" floral buds, with the stigma protruding out of bud during its development (Fig. 4a). However, CS-T18 and CS-M18 produced normal buds like TM-1 without such open bud characteristics suggesting the substituted alleles were functionally similar to TM-1 in these CS lines. Previous studies reported that the locus *Open bud-1* (*ob-1*) is associated with Y_2 in the chromosome 18, complementary mutations exist in *G. hirsutum* (*Ob-1 ob-2*) and *G. darwinii* (*ob-1 Ob-2*) (Endrizzi and Nelson 1989; Endrizzi and Ray 1991). The *ob-1* was previously recovered as an introgression product from *G. darwinii* (Endrizzi and Nelson 1989), a Galapagos Island sibling species of *G. barbadense*, so our finding that CS-B18 is afflicted by the open-bud phenotype is not unexpected.

Plants of CS-T06 were pilose and produced fertile flowers with unfurled petals (Fig. 4b). In both Mississippi and Texas, CS-T06 line produced unopened flowers over most of the flowering time (observed in two successive years), but towards the end of flowering, some flowers opened more or less normally. This effect on flower opening is likely due to increased hairiness of the petal, with various interactions of plant growth, turgor, humidity and so on. *Gossypium tomentosum*, endemic only to the Hawaiian Islands, is one of the most heat-resistant species of the genus and produced normal open flower (Percival et al. 1999; Akhtar et al. 1996). Our results revealed that this feature is only associated with the substituted alien chromosome six (A genome) from *G. tomentosum* in CS-T06 line, but absent in the donor parent of *G. tomentosum*. However, flower petals of *G. tomentosum* are much smaller and so the velcro-like effects may be proportionately much smaller. It will be interesting to investigate in future to understand if this phenotype is due to physical effects and/or genetic interactions. At this time, we do not know if the closed flower phenotype promotes self pollination or not, and/or if it modifies tolerance to heat, or would be sufficient to protect pollen from water droplets, e.g., from irrigation or rain. A trait that promotes self

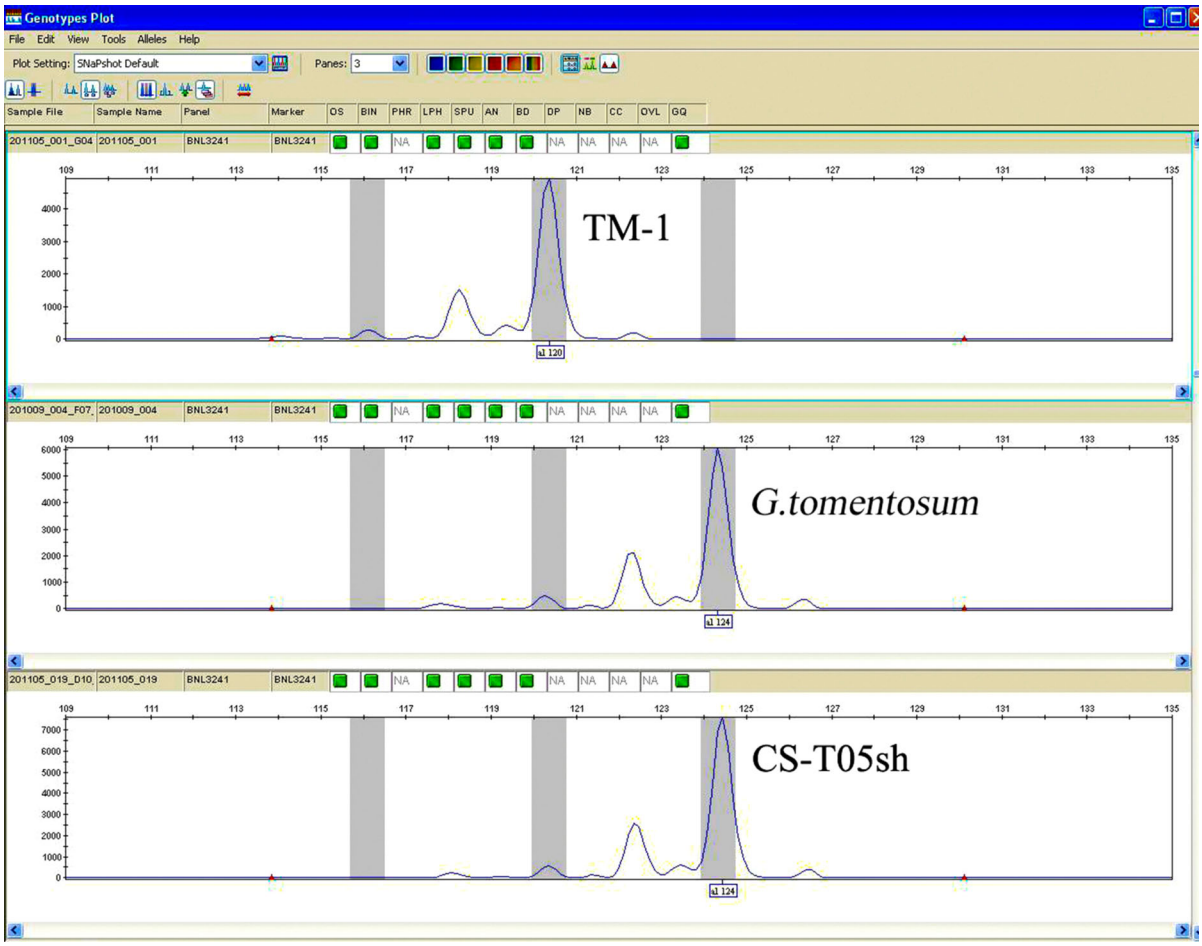

Fig. 1 Electropherograms results by ABI3100 capillary electrophoresis showing CS-T05sh line to be homozygous for *G. tomentosum* for BNL3241 SSR marker specific to chromosome five, but missing the allele from *G. hirsutum* line TM-1. The molecular results illustrate confirmation that the genome CS-T05sh *line* contains a substituted short-arm segment from chromosome 5 from *G. tomentosum*, and lacks the homologous segment from TM-1

Fig. 2 Comparative pictures of TM-1 with the CS lines from three species showing similarities and differences in overall phenotypes

Fig. 3 The recurrent parent TM-1, 3–79 and *G. tomentosum* and *G. mustelinum* plant (the donor parents for the substituted chromosomes or chromosome segments). **a** TM-1, **b** 3–79 (*G. barbadense*), **c** boll of *G. tomentosum* showing *brownish* fiber, **d** flower of *G. tomentosum,* **e** flower of *G. mustelinum,* **f** plant of *G. mustelinum* plant in wild habitat. (Color figure online)

Table 2 Morphological and reproductive characteristics of specific chromosome substitution (CS) lines

Sl No.	CS line	Morphological characters of the CS-lines
1	CS-B02	Big size boll
2	CS-M04	Very late flowering and maturity; taller plant than CS-T04, CS-B04
3	CS-T04	Early maturity with more open boll than CS-M04 and CS-B04
4	CS-B04	Shorter plant than CS-M04 and CS-T04
5	CS-T06	Later flowering than CS-M06 and CS-B06; more glaburous than CS-M06, CS-B06 and TM-1; more insect infestation than CS-M06 and CS-B06, very late maturity with few open boll; fiber more brown color compared to CS-M06 and CS-B06, most flower cleistogamyous type but produces open flowers late in the season
6	CS_B08sh	Shorter plant than CS-M08sh, CS-T08sh and TM-1
7	CS-M11sh	Very late flowering and maturity; taller than CS-T11sh and CS-B11sh; hairy
8	CS-B11sh	Very late flowering and maturity compared to TM-1 and CS-T11sh
9	CS-M22sh	Late flowering and maturity compared to CS-T22sh, CS-B22sh and TM-1
10	CS-T26Lo	Late flowering and maturity; tall plant
11	CS-B26Lo	Very late flowering and maturity; very tall plant
12	CS-M 16-15	Shorter plant than CS-T 16-15 and TM-1

pollination and reduces cross-pollination might be useful to protect transgenic cotton lines from contamination and/or transfer of pollen to neighboring non-transgenic cotton. We also observed that CS-B10-19, developed from a translocation-derived tertiary monosomic (NTN10R-19R), produced deep golden-yellow pollen like the donor parent 3–79 (Fig. 4c). However, the analogous substitutions from *G. tomentosum* and *G. mustelinum*, CS-T10-19 and CS-M10-19, produced cream-colored pollen like TM-1. It has been reported that the tetraploid cotton species produces pollen from cream to deep golden yellow and the true yellow color pollen produced by the genotype of $P_1P_1P_2P_2$ (Endrizzi et al. 1985). Moreover, the P_1 gene was mapped to chromosome 5 (Endrizzi and Ramsay 1980), which is a segmental homeolog of chromosome 19, so the association with CS-B10-19 indicates that a part of the substituted

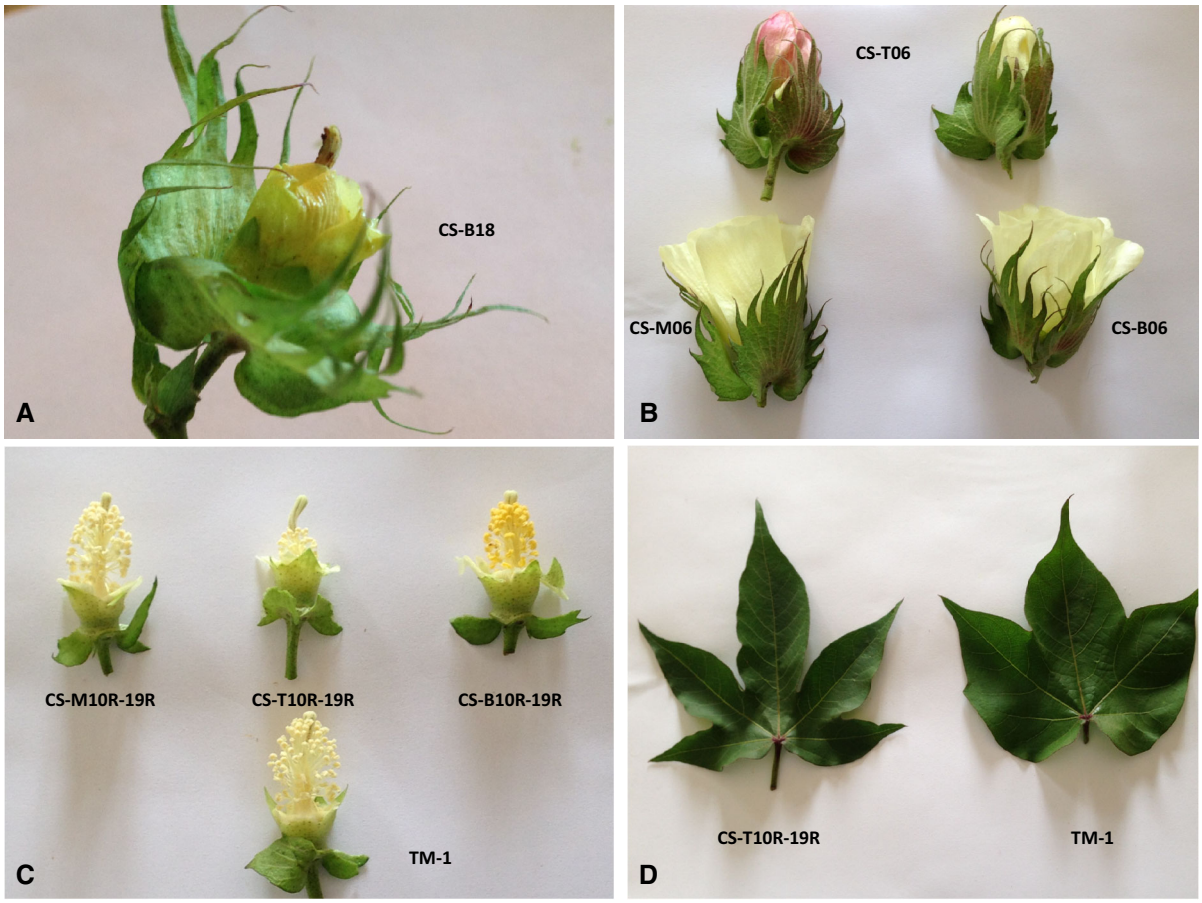

Fig. 4 Some specific characteristics associated with the CS lines: **a** Open-bud mutant with protruding stigma from CS-B18, **b** a cleistogamous type flower from CS-T06, **c** *yellow* pollen from CS-B 10R–19R, **d** leaf from CS-T 10R–19R. (Color figure online)

segment of *G. barbadense* chromosome-19 is homeologous to chromosome 5. We also observed that most of the substitution lines developed from tertiary monosomic stocks had different shape of the lobe on the leaf compared to TM-1 parent (Fig. 4d). This observation presumably reflects the fact that most of the tertiary monosomic recurrent parents were derived from a non-TM-1 background and were not as isogenic with TM-1 as other CS lines. It could be that lobing on the leaf was not due the substituted chromosomes.

In conclusion, we confirmed the cytological results of the following CS lines using chromosome specific SSR markers: CS-B01, CS-B02, CS-B03, CS-B04, CS-B08sh, CS-B09, CS-B10, CS-B11sh_A, CS-B11sh_B, CS-B12, CS-B14sh, CS-B16, CS-B17, CS-B18, CS-B22Lo, CS-B22sh, CS-B25, CS-B26Lo_A, CS-B26Lo_B, CS-B04-15, CS-B10-19, CS-B12-19, CS-B16-15, CS-B17-11, CS-T01, CS-T02, CS-T04-15, CS-T05sh, CS-T06, CS-T07, CS-T10, CS-T10-19, CS-T11sh, CS-T14sh, CS-T16-15, CS-T17, CS-T18, CS-T22sh, CS-M02, CS-M04, CS-M04-15, CS-M06, CS-M08sh, CS-M11sh, CS-M15sh, CS-M16-15, CS-M22sh. Our results documented the development of CS lines from *G. tomentosum* and *G. mustelinum* for the first time. These CS lines can be used as an alternative approach to complement conventional pedigreed or population-based interspecific introgression inducing recombination specific only to the targeted substituted chromosomes or chromosome segments, thereby reducing the linkage drag effects in the genetic improvement of Upland cotton. Our previous studies revealed that many of these chromosome substitution lines are associated with economically important fiber traits (Saha et al. 2011, 2013). We also demonstrated that chromosome substitution lines would be very useful

for improvement of fiber traits in Upland cultivars (Jenkins et al. 2006, 2007). We observed that molecular results with some CS lines are discordant according to our expectation for the donor alleles and further investigation is in progress to discern the underlying basis of these results. Results also showed that some of the CS lines had some specific morphological characteristics compared to the recurrent parents and CS lines for the same substituted chromosome or chromosome segment from other species suggesting that this phenotype was associated with the substituted chromosome or chromosome segment.

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