Euphytica (2018) 214:35
https://doi.org/10.1007/s10681-018-2118-2

Overcoming obstacles to interspecific hybridization between *Gossypium hirsutum* and *G. turneri*

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Received: 14 June 2017 / Accepted: 20 January 2018 / Published online: 29 January 2018
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Abstract *Gossypium turneri*, a wild cotton species ($2n = 2X = 26$, $D_{10}D_{10}$) originating from Mexico, possesses invaluable characteristics unavailable in the cultivated tetraploid cotton gene pool, such as caducous involucels at anthesis, resistance to insects and tolerance to abiotic stresses. However, transferring desired characteristics from wild species into cultivated cotton is often fraught with diverse obstacles. Here, *Gossypium hirsutum* (as the maternal parent) and *G. turneri* were crossed in the Hainan Province of China, and the obtained hybrid seeds ($2n = 3X = 39$, ADD_{10}) were treated with 0.075% colchicine solution for 48 h to double the chromosome complement in order to overcome triploid F_1 sterility and to generate a fertile hexaploid. Chromosome doubling was successful in four individuals. However, the new synthetic hexaploids derived from these individuals were still highly sterile, and no seeds were generated by selfing or crossing. Therefore, an embryo rescue technique was employed in an attempt to produce progenies from the new synthetic hexaploids. Consequently, a total of six large embryos were obtained on MSB2K medium supplemented with 0.5 mg l^{-1} KIN and 250 mg l^{-1} CH using ovules from backcrossing that were 3 days post-anthesis. Four grafted surviving seedlings were confirmed to be the progenies (pentaploids) of the new synthetic hexaploids using cytological observations and molecular markers. Eight putative fertile individuals derived from backcrossing the above pentaploids were confirmed using SSR markers and generated an abundance of normal seeds. This research lays a foundation

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for transferring desirable characteristics from *G. turneri* into upland cotton.

Keywords Chromosome doubling · Embryo rescue · *Gossypium hirsutum* · Interspecific hybridization · Molecular marker · Wild cotton

Introduction

Cotton belongs to the *Gossypium* genus of Malvaceae, which contains five tetraploid species (2n = 4x = 52, AADD) and approximately 45 diploid species (eight genomes from A to G and K, 2n = 2x = 26) (Fryxell 1992). There are eight genomes in the diploid species, designated A to G and K based on chromosomal pairing performance and geographical distribution (Wendel 1989). Upland cotton, *Gossypium hirsutum* L (2n = 52, AADD), which originates from Mesoamerica, is one of four independently domesticated *Gossypium* species (Brubaker et al. 1999). It is the most widely cultivated species, and its fiber yield accounts for over 95% of the world's cotton production (Chen et al. 2007). During its history of improvement, upland cotton was subjected to long-term artificial selection, which greatly narrowed its genetic base and gave rise to challenges in breeding. Consequently, cotton breeders face a scarcity of genetically diverse resources and have attempted to expand the genetic base of cotton cultivars using various methods. Interspecific hybridization of distant species could be an efficient method of broadening the genetic base in cotton. Wild diploid species have many excellent characteristics and contain an abundance of desirable genes; however, these characteristics have yet to be unlocked by pre-breeding. Until now, only a few species have been extensively used in breeding, such as *Gossypium thurberi* and *Gossypium raimondii*, despite the feasibility of several species being explored through numerous breeding programs that investigated, for example, their ability to improve fiber quality and lint yield and enhance resistance to biotic (diseases and pests) and abiotic stresses (low or high temperature, drought, and salinity). One of the most successful examples of the use of a wild species in the history of cotton breeding is *G. thurberi* (2n = 2x = 26, D_1D_1), which was used as a source of fiber quality genes in upland cotton (Culp et al. 1973, 1979). *G. harknessii* (2n = 2x = 26, $D_{2\text{-}2}D_{2\text{-}2}$), which is used as a source of cytoplasmic male sterility genes, has also been extensively studied worldwide (Meyer 1975). *G. sturtianum* and *G. bickii* have been used as donor parents in the development of high-gossypol cotton plants with low-gossypol seeds (Benbouza et al. 2010; Vroh et al. 1999; Zhu et al. 2004; Zhu and Chen 2005). More recently, attempts to introduce other important traits into upland cotton from *G. anomalum* (2n = 2x = 26, B_1B_1), such as longer fiber length and resistance to disease, were made using various strategies (Qian et al. 1992; Wang et al. 2016; Zhai et al. 2015). Genes associated with resistance to the nematode *Rotylenchulus reniformis* were introgressed into upland cotton from *G. longicalyx* and *G. aridum* (Robinson et al. 2007; Romano et al. 2009; Sacks and Robinson 2009). Despite these successes, most of the genetic variation in wild *Gossypium* species remains to be exploited due to interspecific incompatibility.

Gossypium turneri Fryxell, a wild diploid cotton species (2n = 2x = 26, $D_{10}D_{10}$) that originates from Mexico (Fryxell 1978), possesses invaluable characteristics unavailable in the cultivated tetraploid cotton gene pool, such as caducous involucels (or bracts) at anthesis (i.e., bracts that do not remain in lint fibers when harvested, leading to a lower lint impurity content), resistance to insects and tolerance to abiotic stresses. In this study, an interspecific hybridization between *G. hirsutum* and *G. turneri* was achieved, and the obtained seeds were treated with 0.075% colchicine for 48 h to double the F_1 chromosomes in order to overcome triploid F_1 sterility and to produce a fertile hexaploid ($AADDD_{10}D_{10}$). However, we found that the synthetic hexaploid plants were still highly sterile and produced few seeds by self- or cross-pollination. Using an embryo rescue technique, embryos 3 days post-anthesis (dpa) from the cross, *G. hirsutum* × (*G. hirsutum* × *G. turneri*)^2 F_1 (6x), were incubated on MSB2K medium (Sacks 2008) supplemented with 0.5 mg l^{-1} KIN and 250 mg l^{-1} CH to rescue young embryos. Putative backcross hybrid (*G. hirsutum* × *G. turneri*) BC_1F_1 (5x) plants were then produced and characterized using SSR markers and cytological and morphological techniques. Finally, fertile BC_2 progenies derived from backcrossing the above pentaploids with *G. hirsutum* were produced. This research lays the foundation for the transfer of desirable characteristics of *G. turneri* into upland cotton.

Materials and methods

Materials

Gossypium hirsutum acc. TM-1 ($2n = 4x = 52$, AADD), a genetic standard line, was employed as a maternal parent that had been self-pollinated more than 60 times. Diploid wild species of *G. turneri* ($2n = 2x = 26$, $D_{10}D_{10}$), which are native to Mexico, were used as paternal parents, whose pollen was kindly provided by Hainan Wild Cotton Growing Garden, Cotton Research Institute, Chinese Academy of Agricultural Sciences.

Methods

Interspecific hybridization

G. hirsutum flowers were manually emasculated 1 day before anthesis, and the stigmas were covered with a folded plastic straw 4–6 cm in length to prevent cross-hybridization. On the day of anthesis, the emasculated flowers were pollinated with pollen from *G. turneri* under natural field conditions in Hainan Province, China, and the pistils were covered with folded straws again. The growth regulator, gibberellin (GA_3, 50 mg l^{-1}), was then applied to the base of the pedicels of the pollinated flowers to prevent shedding, as described by Qian et al. (1992).

Colchicine treatment

In the period of 2007–2008, to determine suitable conditions for cotton seed chromosome doubling, a series of concentrations of colchicine solution from 0.050 to 0.200% were used to treat the dry seeds of *G. hirsutum* acc. TM-1 for 12, 24 and 48 h. Next, a semi-lethal dose was selected to treat the seeds of interspecific hybrids in order to double the chromosome number. Finally, the obtained interspecific hybrid seeds of *G. hirsutum* × *G. turneri* were treated with 0.075% colchicine solution for 48 h and then rinsed in running water for 10 min before being planted in nursery pots. When they grew seven leaves, the seedlings were transplanted into ceramic pots at the Pailou Experimental Station, Nanjing Agricultural University (PES/NAU). During the winter, all plants were moved into a greenhouse.

In vitro culture

Young bolls 3 days post-anthesis (dpa) were harvested from the cross of *G. hirsutum* × [*G. hirsutum* × *G. turneri* (6x)], washed with soapy water and rinsed in clean water for 20 min. The surfaces of the bolls were then sterilized by immersion in 70% ethanol for 1 min, followed by soaking in 3% $NaHClO_3$ for 10 min in a laminar flow hood. The sterilized bolls were then rinsed three times with distilled water, and then ovules were excised under sterile conditions. The culture media used were as described by Chen et al. (2015). The fresh excised ovules were incubated on ovule enlargement medium MSB2 K (MS with B5 vitamins supplemented with 1.9 g l^{-1} additional KNO3) containing 0.5 mg l^{-1} kinetin (KIN) and 250 mg l^{-1} casein hydrolysate (CH) for 60 days under dark conditions and then transferred to MSB2K medium without KIN and CH for ovule germination. 2 or 3 weeks later, germinated embryos were separated from ovules and cultured in MSB2K + 1.0 mg l^{-1} KIN to encourage seedling growth. Seedlings were cultured in MSB medium containing 0.5 mg l^{-1} α-naphthyl acetic acid (NAA) and 1.0 g l^{-1} AC to allow root formation and MSB medium containing 30 g l^{-1} glucose for healthy growth. Plantlets were then grafted to a stock of disease-resistant cotton, *G. barbadense*. All embryo cultures were grown in a growth chamber with 1600–2000 lux of illumination intensity under a 16/8 h light/dark and 26/24 °C day/night temperature regime.

Morphological observations

Morphological traits were observed for all cotton plants in full bloom, with the exception of *G. turneri*. *G. turneri* had grown for several years in Hainan Province, but no seeds were harvested by self-pollination. In the winter of 2013, these plants were destroyed by a typhoon and were lost. Therefore, no morphological data were obtained from *G. turneri*.

Chromosomal configuration observations and pollen viability test

Young buds, approximately 2–3 mm long, were used for meiotic metaphase chromosome preparations. Young flower buds were collected between 8:30 and 10:00 am. Meiotic chromosome spreads were

prepared as described by Wang et al. (2006) with several modifications. Upon removal of the calyx and corolla, the collected buds were fixed in ethanol-acetic acid (3:1, v/v) fixative for 2–24 h at 4 °C. Next, buds containing pollen mother cells (PMC) in metaphase I were selected, and several anthers from the selected buds were placed onto ethanol-washed glass slides with a drop of 45% acetic acid (v/v), freed of debris and squashed. The slides were examined under an Olympus BX51 fluorescence microscope. The slides were stained with 4′,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics, Mannheim, Germany) for 10 min at room temperature, and anti-fade (Vector, USA) was applied under the coverslips. Pollen fertility was tested with a 1% I-KI solution. Chromosome and pollen viability images were captured using an evolution VF CCD camera (Media Cybernetics, Bethesda, MD, USA), and image processing was performed using Image-Pro express software (Media Cybernetics, Bethesda, MD, USA).

SSR molecular marker characterization

Total genomic DNA was extracted from young leaves of the parent, *G. hirsutum*, and the obtained hybrid plants (F_1, BC_1) as described by Paterson et al. (1993). The DNA of *G. turneri* was kindly provided by Dr. Fang, Crop Genetics Research Unit, USDA-ARS. Based on the high-density tetraploid cotton linkage map constructed in our laboratory (Guo et al. 2007), 1845 pairs of simple sequence repeat (SSR, microsatellite) primers were selected at intervals of approximately 3–5 cM to screen for polymorphisms between the two parents. Polymorphic primers were used for the identification of their interspecific hybrid F_1 progenies and the backcross progenies. All SSR primer information for this work can be downloaded from https://www.cottongen.org. PCR amplification was performed in a volume of 10 μl containing 1 μl of DNA extract (20 ng μl^{-1}), 1 μl of 5.0 μM of each primer, 1 μl of 2.50 mM $MgCl_2$, 0.2 μl of 200 μM dNTPs, 0.1 μl of Taq polymerase (5 U μl^{-1}), 1 μl of reaction buffer (10x) and 4.7 μl of ddH_2O. SSR-PCR amplifications were performed using a Peltier Thermal Cycler EDC-810 (Eastwin, Hong Kong), and electrophoresis of the PCR products was performed as described by Zhang et al. (2002).

Results

Production of the hexaploid of *G. hirsutum* (2n = 4X = 52, AADD) × *G. turneri* (2n = 2X = 26, $D_{10}D_{10}$) by colchicine treatment

In the period of 2007–2008, to determine suitable conditions for cotton seed chromosome doubling, a series of concentrations of colchicine solution, 0.050, 0.075, 0.100 and 0.200%, was used to treat 20 dry seeds of *G. hirsutum* acc. TM-1 for 12, 24 and 48 h. The results indicated that the higher the concentration of colchicine solution and the longer the treatment time, the fewer seedlings survived. A semi-lethal dose for chromosome doubling was presumed to be suitable (Table 1). Therefore, treatment with 0.075% colchicine solution for 48 h was selected for chromosome doubling of interspecific hybrids.

Under the above conditions, thirty putative interspecific hybrid F_1 seeds of *G. hirsutum* (2n = 4x = 52) × *G. turneri* (2n = 2x = 26) were treated in 2008, from which twelve individuals were obtained. The individuals were examined for their chromosome configurations in meiosis, morphological traits, and SSR markers. The SSR markers showed that all 12 plants were interspecific hybrid F_1 offspring of *G. hirsutum* × *G. turneri*. Moreover, morphological observations demonstrated that four of the 12 interspecific hybrid plants treated with colchicine were putative hexaploid plants, and this was further confirmed by investigation of their chromosomes (2n = 6X = 78, $AADDD_{10}D_{10}$). All pollen mother cells (PMCs) from the undoubled plants had 39 chromosomes, and the chromosome configurations were variable, with 14.27 univalents, 8–12 bivalents and 1–2 trivalents (Fig. 1; Table 2). The average chromosome configurations were 2.67 uni-, 11.16 bi- and 0.78 trivalents.

The chromosome configurations of the plants treated with colchicine are shown in Fig. 2. The results showed that the number of univalents decreased while the number of bivalents greatly increased, which indicated that the chromosomes of these four plants were doubled, although it was hard to clearly count the chromosome numbers in PMCs due to the small sizes of numerous chromosomes. In PMCs at meiosis, however, chromosome bridges and lagging chromosomes were also observed in the putative chromosome-doubled plants. To further verify the

Table 1 Cotton seeds were treated with different colchicine concentrations for different lengths of time

Time/h	Colchicine concentration (%)	No. seeds treated	No. seedlings survived	No. variants	Percentage of variants (%)
12 h	0.050	20	20	3	15.00
	0.075	20	19	3	15.79
	0.100	20	19	4	21.05
	0.200	20	15	4	26.67
24 h	0.050	20	19	5	26.31
	0.075	20	19	5	26.31
	0.100	20	16	5	31.25
	0.200	20	12	4	33.33
48 h	0.050	20	15	5	33.33
	0.075	20	10	5	50.00
	0.100	20	6	3	50.00
	0.200	20	5	3	60.00

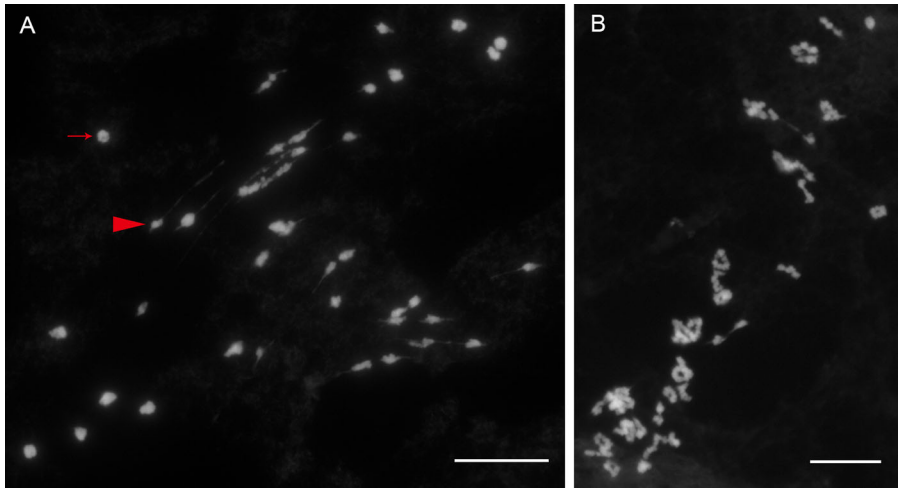

Fig. 1 Chromosome configuration at metaphase I of meiosis in pollen mother cells. **a** The triploid interspecific hybrid F_1 of *G. hirsutum* × *G. turneri* (2n = 3X = A DD_{10}). Red arrows indicate univalents, and arrowheads indicate bivalents. **b** Many bivalents are present in the hexaploid of *G. hirsutum* × *G. turneri* (2n = 6X = A $ADD_{10}D_{10}$). Scale bars, 10 μm. (Color figure online)

authenticity of the putative chromosome-doubled plants, their pollen and that from control plants (without colchicine treatment) were stained with 1% I-KI solution to compare their viabilities. The results showed that pollen from the four putative hexaploid plants had much higher viability (more normal, round-shaped pollen grains) than that of the control (Fig. 2; Table 3).

Production of a pentaploid (BC_1F_1) of *G. hirsutum* × *G. turneri* by embryo rescuing

After chromosome doubling of interspecific hybrid F_1 plants, the obtained hexaploid plants (2n = 6X = 78, $AADDD_{10}D_{10}$) of *G. hirsutum* and *G. turneri* were still highly sterile and produced no seeds by selfing or backcrossing during the 7 years following (whether used as paternal or maternal parents). To obtain backcrossed progenies, the embryo rescue technique was improved in this study. First, we backcrossed *G. hirsutum* as the maternal parent with the hexaploid of

Table 2 Chromosome configurations of pollen mother cells at metaphase I of meiosis in PMCs of the interspecific hybrid F_1, *G. hirsutum* × *G. turneri*, 2n = 3x = ADD_{10}

No. PMCs	I	II	III	No. chromosomes
22	14	11	1	39
10	13	13	0	39
8	16	10	1	39
6	12	12	1	39
4	15	12	0	39
3	17	8	2	39
2	18	9	1	39
Range	12–18	8–13	0–2	39
Average	14.27	11.16	0.78	39

G. hirsutum × *G. turneri* as the paternal parent. Fifty mg l^{-1} of gibberellin (GA_3) was then dropped onto the base of the pedicels of the pollinated flowers for three consecutive days, twice a day, to prevent shedding. Next, three-day-old bolls were collected, and ovules were excised from them and cultured on MSB2K medium supplemented with 0.5 mg l^{-1} Kin and 250 mg l^{-1} CH in the dark. A total of 285 intact ovules from 23 bolls were cultured on the initial medium. Of these, 81% remained viable and 67% expanded after 30 days of culture (Table 2). A small number of ovules germinated and six large embryos were isolated. Four embryos developed into seedlings, while the other two died (Fig. 3). After the plantlets developed 3–4 leaves, they were grafted onto a stock of *G. barbadense* for further growth (Table 4).

Morphological characteristics of the obtained putative pentaploid of *G. hirsut*um × *G. turneri* (2n = 5X = $AADDD_{10}$)

The obtained putative pentaploid plants of *G. hirsutum* × *G. turneri* were very similar to each other in morphology (Fig. 4). An investigation of 26 traits of the putative pentaploids was performed, including stem color and hairiness, leaf shape and color, flower size, bract size and fertility (Table 5). The results indicated that the pentaploids were similar to the hexaploid paternal parent in most morphological traits; for example, they had light red stems, dark green leaves, a thick hard leaf texture, a similar leaf length and width, dark green petioles, yellow petals, large flowers, a similar petal length and width, a similar stigma length, yellow anthers, a similar anther number, and a similar pedicel length, and they were sterile. A few traits resembled those of the maternal parent, *G. hirsutum*, such as the petiole length, the presence of petal spots and bracteole dentations, the bracteole dentation number and the bracteole length and width. Other characteristics were shared with both parents, such as stem hairiness, leaf hairiness, shallow

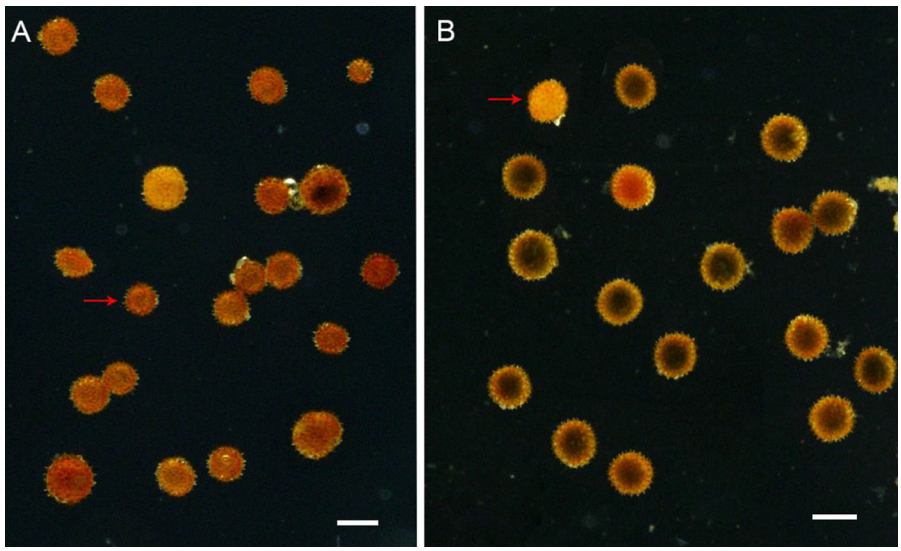

Fig. 2 Pollen of the triploid hybrid F_1 and the hexaploid hybrid F_1 of *G. hirsutum* × *G. turneri*. **a** Abnormal pollen of the triploid hybrid F_1. **b** Normal pollen of the hexaploid hybrid F_1. Red arrows indicate the abnormal pollen. Scale bars, 10 μm. (Color figure online)

Table 3 Pollen fertility of the interspecific hybrid of *G. hirsutum* × *G. turneri* after treatment with 0.075% colchicine for 48 h

Individual	No. observed pollen grains	Pollen fertility (%)
1	365	15.5 ± 3.4
2	360	11.1 ± 2.5
3	356	85.3 ± 3.3
4	321	12.3 ± 3.5
5	350	9.5 ± 2.2
6	363	10.3 ± 5.5
7	354	80.7 ± 3.3**
8	365	87.6 ± 2.6**
9	360	7.8 ± 5.6
10	358	13.2 ± 3.1
11	353	10.2 ± 2.3
12	354	83.4 ± 2.1**

** Stands for the significant level $P = 0.01$

lobed leaves, the leaf lobe number and creamy stigma. Based on the above morphological traits, the putative pentaploids of *G. hirsutum* × *G. turneri* were confirmed to be the progenies of the hexaploid backcrossed with *G. hirsutum*.

Cytological observation of the obtained putative pentaploid of *G. hirsutum* × *G. turneri* ($2n = 5X = AADDD_{10}$)

Cytological observations of the putative pentaploid of *G. hirsutum* × *G. turneri* ($2n = 5x = AADDD_{10}$) are shown in Table 6. All of the cells observed had 65 chromosomes, indicating that they were pentaploid ($2n = 5x = 65$; Fig. 5). The results also verified that the pentaploids were derived from the progenies of the hexaploid of *G. hirsutum* × *G. turneri* ($2n = 6x = 78$) backcrossed with *G. hirsutum*. The chromosome configurations of the pentaploids in meiosis were variable, with uni-, bi- and trivalents. The chromosome configurations of 51 cells were clearly discriminated. Most cells (18/51) showed eight univalents, 21 bivalents and five trivalents, followed by cells (15/51) with seven univalents, 20 bivalents and six trivalents. The average chromosome configurations were 7.47

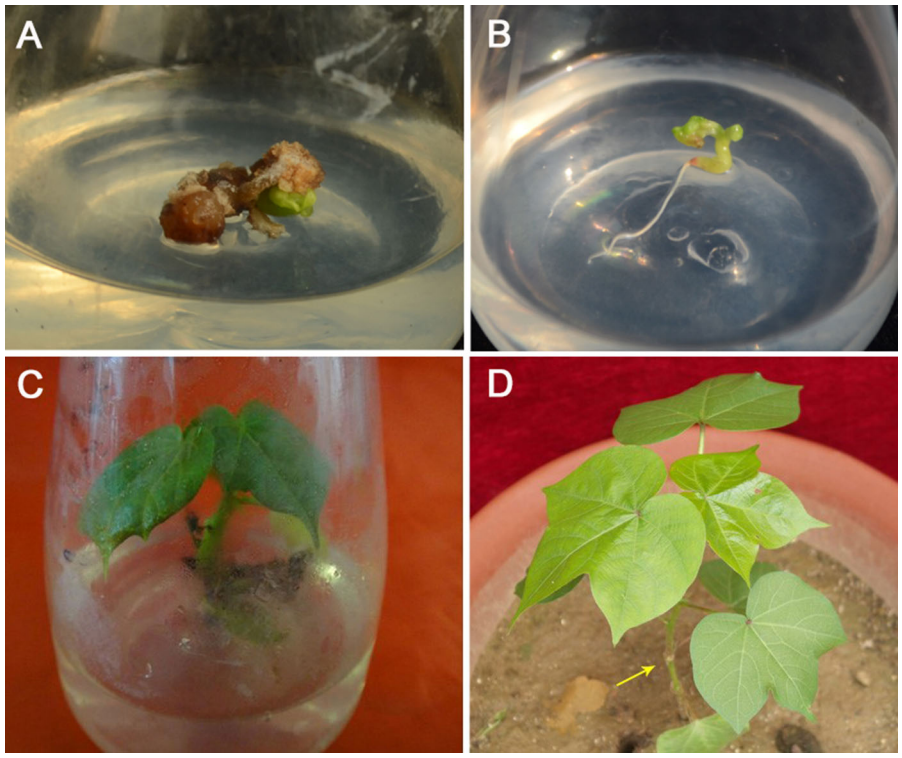

Fig. 3 Embryo rescue of [*G. hirsutum* × (*G. hirsutum* × *G. turneri*)^2] F_1 hybrid ovules. **a** Germinated ovule. **b** Isolated embryo. **c** Healthy plantlets. **d** Grafted plantlets

Table 4 The result of ovule culture of [*G. hirsutum* × (*G. hirsutum* × *G. turneri*)^{2}F$_1$] (5x)

No. bolls	No. obtained ovules	Ovule viability (%)	Embryo expanded (%)	No. germinated embryos	No. plants
23	285	81	67	6	4

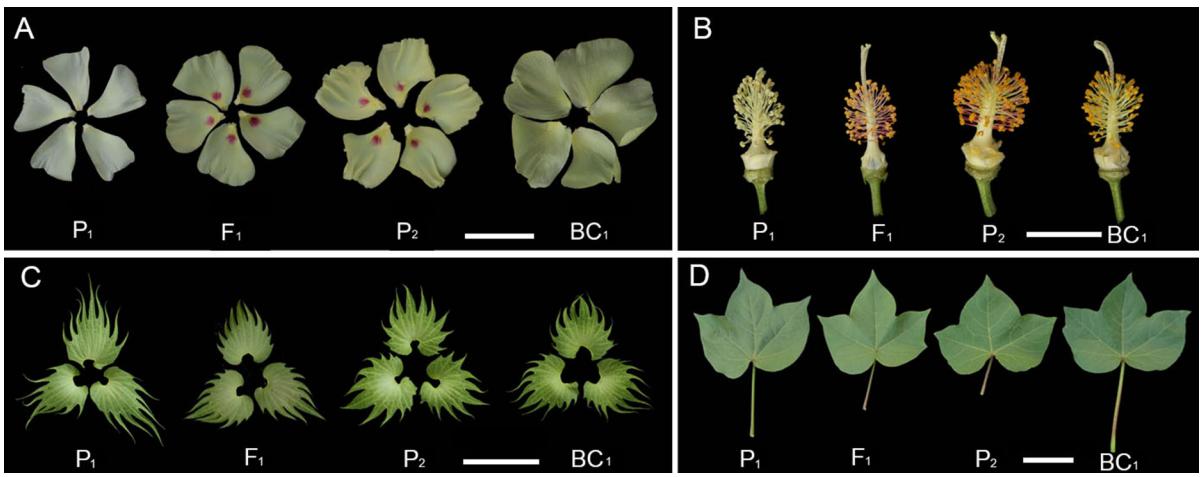

Fig. 4 Flowers of *G. hirsutum*, (*G. hirsutum* × *G. turneri*) F$_1$ (3X), (*G. hirsutum* × *G. turneri*) F$_1$ (6x) and [*G. hirsutum* × (*G. hirsutum* × *G. turneri*) ^{2}] F$_1$ (5x). **a** Petals. **b** Pistils and stamens. **c** Bracts. **d** Leaves. Scale bars, 30 mm. P$_1$. *G. hirsutum* acc. TM-1; F$_1$. (*G. hirsutum* × *G. turneri*) F$_1$ (3x); P$_2$. (*G. hirsutum* × *G. turneri*) F$_1$ (6x); BC$_1$. [*G. hirsutum* × (*G. hirsutum* × *G. turneri*) ^{2}] F$_1$ (5x)

uni-, 20.47 bi- and 5.53 trivalents. The number of univalents ranged from five to eight, with eight being the most frequent number, followed by seven and nine. The number of bivalents ranged from 18 to 21, with 21 being the most frequent number, followed by 20 and 22. The high frequency of univalents at metaphase I in PMCs in meiosis explained why the pentaploid (2n = 5X = 65) plants were sterile: univalents commonly give rise to disordered segregation and are often lost at anaphase I, leading to the formation of unbalanced and nonviable gametes lacking a complete set of chromosomes.

Reconfirmation of the obtained pentaploid of *G. hirsutum* × *G. turneri* (2n = 5X = AADDD$_{10}$) using SSR markers

We used 1845 SSR primer pairs/combinations selected from the linkage maps of the *G. hirsutum* and *G. barbadense* genomes constructed at our institute (Guo et al. 2007) to screen for polymorphic primers between *G. hirsutum* and *G. turneri*. Approximately 52.3% (960/1845) of the SSRs showed polymorphisms between these two species. Of the 960 pairs of polymorphic primers used to characterize the putative backcross progenies, 513 (53.4%) were dominant alleles in *G. hirsutum*, whereas 400 (41.7%) were codominant alleles in the hybrids, and 47 (4.9%) were dominant alleles in *G. turneri*. Figure 6 and Table 7 show the polymorphic primers that were nearly evenly distributed on the *G. turneri* chromosomes. The amplicons generated by codominant primers in the hybrids demonstrated that the hybrids had DNA bands from both parents (Fig. 7), reconfirming that the putative pentaploids were derived from the progenies of a hexaploid of *G. hirsutum* × *G. turneri* (2n = 6X = AADDD$_{10}$D$_{10}$) backcrossed with *G. hirsutum*.

Discussion

Cotton bract debris is one of the main sources of contamination in mechanically picked cotton and can seriously impair the cleanliness of cotton. *G. turneri* Fryxell (2n = 2x = 26, D$_{10}$D$_{10}$) has caducous bracts

Table 5 Morphological characteristics of *G. hirsutum* × *G. turneri*

Traits	TM-1	Triploid (3x)	Hexaploid (6x)	Pentaploid (5x)
Stem color	Green	Light red	Light red	Light red
Stem hairiness	Light pubescent	Light pubescent	Light pubescent	Light pubescent
Leaf color	Green	Green	Dark green	Dark green
Leaf hairiness	Hairy	Hairy	Hairy	Hairy
Leaf shape	Shallow lobed	Shallow lobed	Shallow lobed	Shallow lobed
Leaf lobe number	2–4	2–4	2–4	2–4
Leaf texture	Thin soft	Thick hard	Thick hard	Thick hard
Leaf length (cm)	11.32 ± 1.16	9.53 ± 0.59	11.12 ± 0.50	12.88 ± 0.54
Leaf width (cm)	14.48 ± 1.65	13.07 ± 0.19	16.33 ± 0.40	16.28 ± 0.43
Petiole color	Green	Green	Dark green	Dark green
Petiole length (cm)	9.97 ± 1.36	7.96 ± 0.63	7.41 ± 0.69	10.09 ± 0.79
Petal color	Creamy	Yellow	Yellow	Yellow
Flower size	Medium	Medium	Large	Large
Petal spot	Absent	Dark red	Dark red	Absent
Petal length (cm)	4.85 ± 0.27	5.02 ± 0.16	5.75 ± 0.38	5.92 ± 0.28
Petal width (cm)	4.98 ± 0.27	5.38 ± 0.21	6.39 ± 0.56	6.79 ± 0.35
Stigma color	Creamy	Creamy	Creamy	Creamy
Stigma length (cm)	1.00 ± 0.13	1.71 ± 0.56	2.14 ± 0.25	1.9 ± 0.18
Anther color	Creamy	Yellow	Yellow	Yellow
Anther number	121.50 ± 4.67	134.10 ± 4.87	132.50 ± 10.70	131.92 ± 5.57
Female fertility	Fertile	Sterile	Sterile	Sterile
Pollen viability	Viable	Unviable	Viable	Viable
Bracteole dentation shape	Long	Short	Short	Long
Bracteole dentation number	10–13	8–11	8–11	10–13
Bracteole length (cm)	5.04 ± 0.39	3.72 ± 0.22	3.73 ± 0.66	4.52 ± 0.30
Bracteole width (cm)	3.85 ± 0.41	316 ± 0.14	3.78 ± 0.59	4.09 ± 0.23
Pedicel length (cm)	1.7 ± 0.36	1.51 ± 0.34	1.34 ± 0.30	1.23 ± 0.22

Table 6 Chromosome configurations at metaphase I of meiosis in PMCs of the pentaploid, *G. hirsutum* × (*G. hirsutum* × *G. turneri*)^2 (2n = 5x = $AADDD_{10}$)

No. PMCS	I	II	III	No. chromosomes
18	8	21	5	65
15	7	20	6	65
9	9	22	4	65
6	6	19	7	65
3	5	18	8	65
Range	5–9	18–21	4–8	65
Average	7.47	20.47	5.53	65

at anthesis, and this trait can be used to improve commercial cotton and can allow convenient mechanical harvesting methods to continue to be used. However, the introgression of the caducous bract gene from the diploid wild species *G. turneri* into the most important cultivated cotton, *G. hirsutum*, is a time-consuming and daunting task due to interspecific barriers; to date, very few breeders have continued to engage in distant hybridization breeding in cotton using diploid species.

In this study, we crossed *G. hirsutum* and *G. turneri* and obtained 30 seeds in 2007. In the second year, four chromosome-doubled plants were obtained by colchicine treatment of the interspecific hybrid seeds of *G. hirsutum* and *G. turneri* and hexaploid plants were subsequently obtained. However, the hexaploid plants were still highly sterile and produced no seeds by either selfing or backcrossing during the seven successive years (whether used as a paternal or

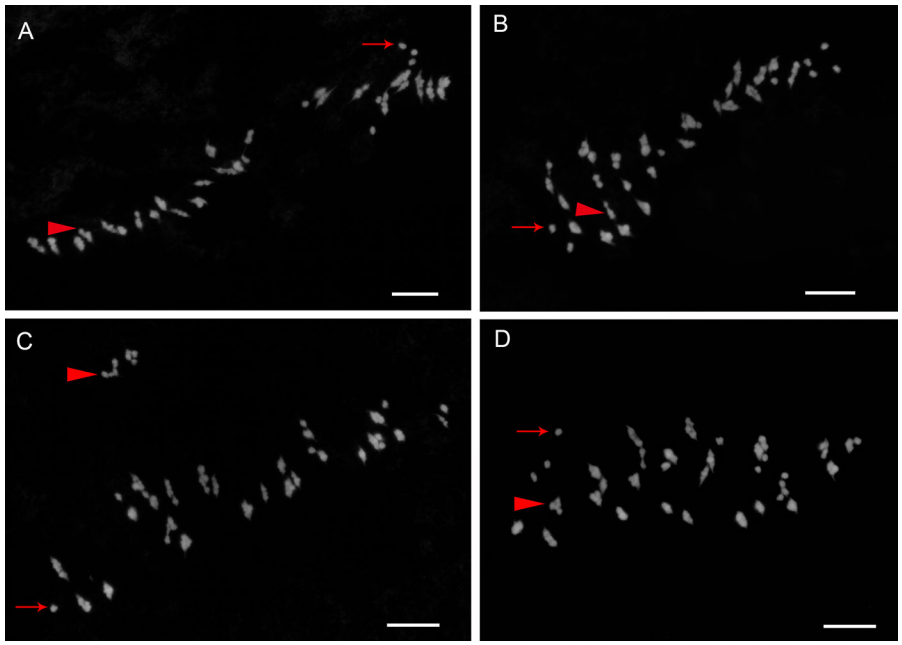

Fig. 5 Configuration at metaphase I of meiosis of pollen mother cells of [*G. hirsutum* × (*G. hirsutum* × *G. turneri*) ^{2}] F1 (5x). **a** 7I + 14II + 10III; **b** 9I + 19II + 6III; **c** 4I + 19II + 7III; **d** 8I + 18II + 7III. Arrows indicate the univalents, and arrowheads indicate the trivalents. Bar = 10 μm

maternal parent). Evaluation of pollen viability showed that the pollen grains geminated on the stigma and produced pollen tubes that descended into the styles and toward the ovary via the transmitting tract as normal (Fig. 8), which indicated that the pollen of the hexaploid plants was viable. However, we peeled the bolls and found that ovules from the hexaploid plants stopped development in the second day post-anthesis, and 4–5 days later, all ovules aborted (Fig. 9). It could be inferred that in the new synthetic hexaploid, chromosome components are 2n = 6x = $AADDD_{10}$-D_{10}, with four sets of D chromosomes, leading to a disorder resulting from early chromosome dissociation or asynapsis in meiosis and subsequently producing imbalanced and poorly viable female gametes. However, we presume that it is more likely that the developmental dysbiosis (misadjustment) occurred between the endosperm and embryo stages. Abnormal endosperm is often formed through interspecific hybridization and results in early decomposition such that subsequent embryos would die due to a lack of nutrition from the endosperm (He et al. 1989, 1991). *G. turneri*, as a wild species, originated from Mexico and was introduced into China. This species produces very few seeds by selfing but can be easily crossed with upland cotton to generate hybrid seeds, implying that the viability of its female gametes was very poor or, more likely, that development dysbiosis (misadjustment) occurred between the endosperm and embryo. The derived hexaploid plants might inherit these traits and show female sterility. This hypothesis remains to be studied.

In addition, although the pollen from the derived hexaploid plants geminated on the stigma and produced pollen tubes that grew into the styles and toward the ovary via the transmitting tract as normal (Fig. 8) when backcrossed with *G. hirsutum*, the dysplastic seeds generated had difficulty germinating, indicating that interspecific obstacles still existed, such as developmental dysbiosis between endosperm and embryo. Therefore, embryo rescue was necessary. Using the embryo rescue technique, we successfully obtained backcross progenies from the hexaploid plants; pentaploids, which were further validated by cytological observation and molecular markers.

The derived pentaploid, however, showed still low fertility and produced few normal seeds when backcrossed with *G. hirsutum* (as the maternal parent). In

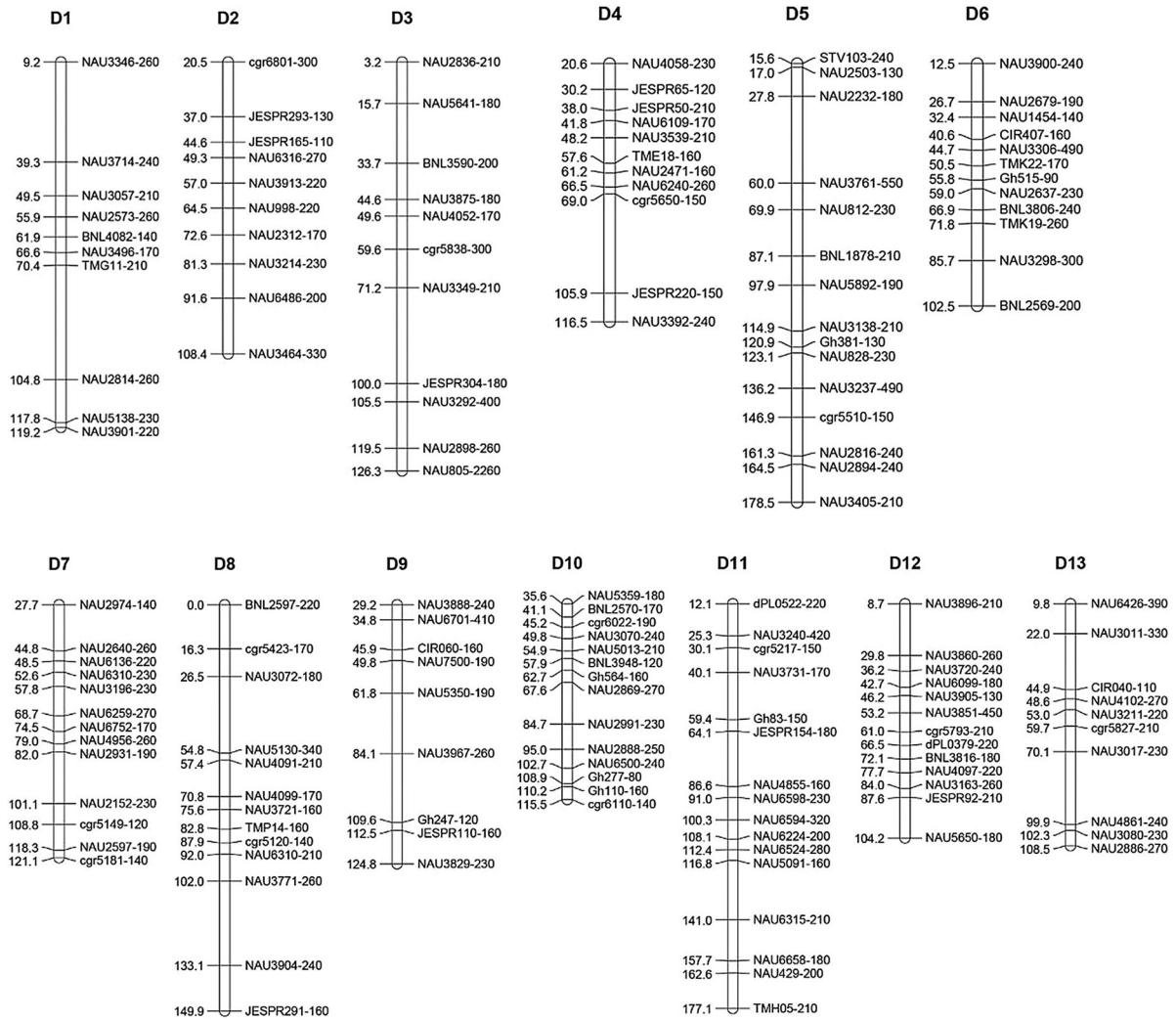

Fig. 6 A putative set of *G. turneri* chromosome-specific SSR markers. Markers are based on the backbone map of the D-subgenome of tetraploid cotton constructed using the BC_1 population of (*G. hirsutum* × G. barbadense) × *G. hirsutum* (Guo et al. 2007). The molecular weight shown is of the amplicon of *G. turneri*

2015, 59 flowers of *G. hirsutum* were emasculated and pollinated with pollen from the derived pentaploid, and 35 seeds were harvested. All the seeds were sown, and 14 seedlings were obtained. Among them, eight were confirmed to be backcross progenies by SSR markers (Fig. 10). The eight individuals were fertile and produced normal seeds, which can be further used to transfer the desirable characteristics from *G. turneri* into upland cotton in the future.

Wild diploid species of *Gossypium* possess an impressive range of variation in many of their characteristics, and all of the characteristics can be exploited in future cotton improvement programs. However, transferring desired characteristics from wild species into cultivated cotton is fraught with diverse obstacles and difficulties. Incompatibility caused by several pre- or post-zygotic barriers is known to limit the production of hybrids between wild diploid species and cultivated cotton. The pre-fertilization barriers encountered in interspecific crossing include failure of pollen germination and slow pollen tube growth. Post-fertilization barriers further hinder the development of the zygote after fertilization and normal development of the seed. These post-

Table 7 *G. turneri*-specific SSR primers screened

Chromosome	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13
	NAU3346-260	cgr6801-300	NAU2836-210	NAU4058-230	STV103-240	NAU3900-240	NAU2974-140	BNL2597-220	NAU3888-240	NAU5359-180	dPL0522-220	NAU3896-210	NAU6426-390
	NAU3714-240	JESPR193-130	NAU5641-180	JESPR65-120	NAU2503-130	NAU2679-190	NAU2640-260	cgr5423-170	NAU6701-410	BNL2570-170	NAU3240-420	NAU3860-260	NAU3011-330
	NAU3057-210	JESPR165-110	BNL3590-200	JESPR50-210	NAU2232-180	NAU1454-140	NAU6136-220	NAU3072-180	CIR060-160	cgr6022-190	cgr5217-150	NAU3720-240	CIR040-110
	NAU2573-260	NAU6316-270	NAU3875-180	NAU6109-170	NAU3761-550	CIR407-160	NAU6310-230	NAU5130-340	NAU7500-190	NAU3070-240	NAU3731-170	NAU6099-180	NAU4102-270
	BNL4082-140	NAU3913-220	NAU4052-170	NAU3539-210	NAU812-230	NAU3306-490	NAU3196-230	NAU4091-210	NAU5350-190	NAU5013-210	Gh83-150	NAU3905-130	NAU3211-220
	NAU3496-170	NAU998-220	cgr5838-300	TME18-160	BNL1878-210	TMK22-170	NAU6259-270	NAU4099-170	NAU3967-260	BNL3948-120	JESPR54-180	NAU3851-450	cgr5827-210
	TMG11-210	NAU2312-170	NAU3349-210	NAU2471-160	NAU5892-190	Gh515-90	NAU6752-170	NAU3721-160	Gh247-120	Gh564-160	NAU4855-160	cgr5793-210	NAU3017-230
	NAU2814-260	NAU3214-230	JESPR304-180	NAU6240-260	NAU3138-210	NAU2637-230	NAU4956-260	TMP14-160	JESPR110-160	NAU2869-270	NAU6598-230	dPL0379-220	NAU4861-240
	NAU5138-230	NAU6486-200	NAU3292-400	cgr5650-150	Gh381-130	BNL3806-240	NAU2931-190	cgr5120-140	NAU3829-230	NAU2991-230	NAU6594-320	BNL3816-180	NAU3080-230
	NAU3901-220	NAU3464-330	NAU2898-260	JESPR220-150	NAU828-230	TMK19-260	NAU2152-230	NAU6310-210		NAU2888-250	NAU6224-200	NAU4097-220	NAU2886-270
			NAU805-2260	NAU3392-240	NAU3237-490	NAU3298-300	cgr5149-120	NAU3771-260		NAU6500-240	NAU6524-280	NAU3163-260	
					cgr5510-150	BNL2569-200	NAU2597-190	NAU3904-240		Gh277-80	NAU5091-160	JESPR92-210	
					NAU2816-240		cgr5181-140	JESPR291-160		Gh110-160	NAU6315-210	NAU5650-180	
					NAU2894-240					cgr6110-140	NAU6658-180		
					NAU3405-210						NAU429-200		
Total	10	10	11	11	15	12	13	13	9	14	16	13	10
Position	9.2–119.2	20.5–108.4	3.2–126.3	20.6–116.5	15.6–178.5	12.5–102.5	27.7–121.1	0.0–149.9	29.2–124.8	35.6–115.5	12.1–177.1	8.7–104.2	9.8–108.5
Mean density[a]	11.0	8.8	11.2	8.7	10.9	7.5	7.2	11.5	10.6	5.7	10.3	7.3	9.9
GDC (cM)[b]	110.0	87.9	123.1	95.9	162.9	90.0	93.4	149.9	95.6	79.9	165.0	95.5	98.7
PCC (%)[c]	87.5	78.5	97.5	75.2	85.8	65.3	72.2	90.4	63.3	69.2	91.1	71.5	84.3

[a]Indicates the genetic distance (cM) between two adjacent markers on a chromosome

[b]GDC means genetic distance coverage (cM)

[c]PCC means the percentage of the chromosome covered by the markers (%)

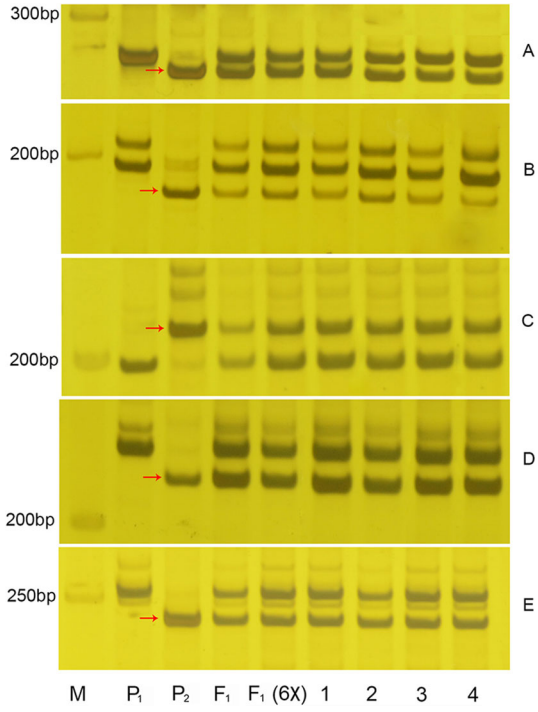

Fig. 7 Validation of the backcrossed progenies derived from the hexaploid of (*G. hirsutum* × *G. turneri*) F_1 (6X) using a putative set of *G. turneri*-specific SSR primers. P_1. *G. hirsutum*; P_2. *G. turneri*; F_1. the triploid interspecific hybrid F_1; F1(6x). the hexaploid interspecific hybrid F_1; 1–4. the individuals derived from the hexaploid interspecific hybrid F_1 of (*G. hirsutum* × *G. turneri*)^2 backcrossed with *G. hirsutum* acc. TM-1; M. molecular marker sizes (50-bp ladder). From **a–e,** *G. turneri*-specific amplicons (arrows) were detected using individual chromosome-specific SSR primers NAU3967, NAU3013, NAU2858, NAU3967 and NAU2580

Fig. 9 The development of ovules of the hexaploid of (*G. hirsutum* × *G. turneri*) F_1 (6x) stopped post self-pollination. 1–9. The days post-anthesis

fertilization barriers include ovaries that do not enlarge after pollination; ovaries that enlarge, but young bolls shed several days post-anthesis; bolls that mature but produce empty seeds or immature seeds that do not germinate; seeds that germinate but die in the cotyledon stage; and crossed plants with high sterility. Our study provides a reference for overcoming interspecific incompatibilities when diploid species are used as donor parents.

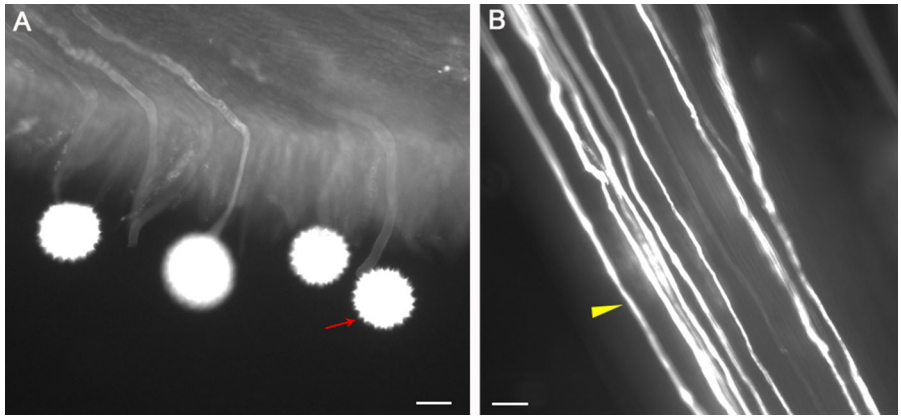

Fig. 8 Pollen germinated on the stigmas of the hexaploid of (*G. hirsutum* × *G. turneri*) F_1 (6x) via self-pollination. **a** Germinated pollen; **b** pollen tubes grew into their own style. Bar = 10 μm

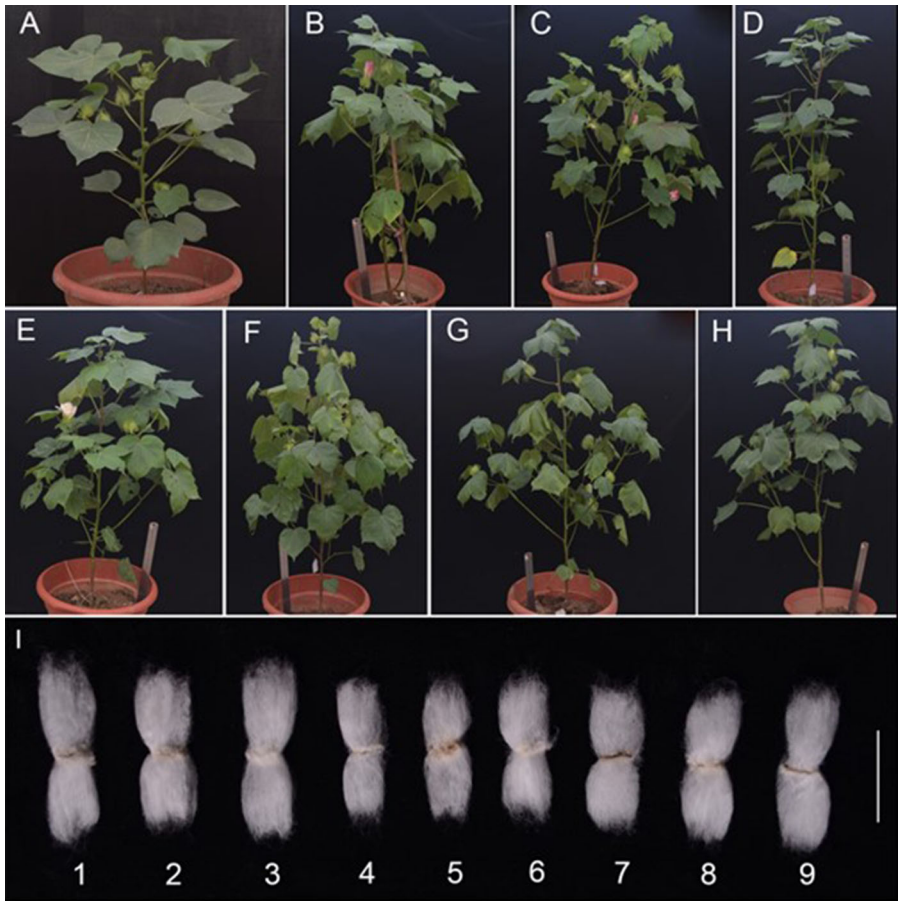

Fig. 10 The backcross progenies (BC_2) derived from the pentaploid of *G. hirsutum* × *G. turneri* backcrossed with *G. hirsutum* acc. TM-1. **a**–**h** The individual plants T17, T21, T29, T30, T31, T36, T39, and T42; 1–9. Fibers of one plant of *G. hirsutum* acc. TM-1, T17, T21, T29, T30, T31, T36, T39, and T42. Bar = 3 cm

Acknowledgements This program was financially supported in part by the National Key Research and Development Program of China (2016YFD0100203), the National Key Technology R&D Program of China during the Twelfth Five-year Plan Period [Grant Number 2013BAD01B03-04] and Jiangsu Collaborative Innovation Center for Modern Crop Production. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. We also acknowledge Dr. Kunbo Wang, Vice Director of Cotton Research Institute of Chinese Academy of Agricultural Sciences, for providing pollen of *Gossypium turneri*. We are grateful to Dr. RJ Kohel of the Southern Plains Agricultural Research Center, USDA-ARS, for providing seeds of *Gossypium hirsutum* acc. TM-1. We also express our thanks to Dr. Fang, Crop Genetics Research Unit, USDA-ARS, for kindly providing the DNA of *G. turneri*.

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