

RESEARCH ARTICLE

A New Sythetic Hybrid (A₁D₅) between *Gossypium herbaceum* and *G. raimondii* and Its Morphological, Cytogenetic, Molecular Characterization

Yuxiang Wu^{1,2}, Di Chen³, Shuijin Zhu^{2*}, Lufei Zhang¹, Lingjiao Li¹

1 College of Agriculture, Shanxi Agricultural University, Taigu, Shanxi, China, **2** Department of Agronomy, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, Zhejiang, China, **3** Cotton Research Institute of Chinese Academy of Agricultural Science, Anyang, Henan, China

☞ These authors contributed equally to this work.

* shjzhu@zju.edu.cn



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Abstract

The diploid species *G. herbaceum* (A1) and *G. raimondii* (D5) are the progenitors of allotetraploid cotton, respectively. However, hybrids between *G. herbaceum* and *G. raimondii* haven't been reported. In the present study, hybridization between *G. herbaceum* and *G. raimondii* was explored. Morphological, cytogenetic and molecular analyses were used to assess the hybridity. The interspecific hybrid plants were successfully obtained. Most of the morphological characteristics of the hybrids were intermediate between *G. herbaceum* and *G. raimondii*. However, the color of glands, anther cases, pollen and corolla, and the state of bracteoles in hybrids were associated with the *G. herbaceum*. The color of staminal columns and filaments in hybrids were associated with *G. raimondii*. Cytogenetic analysis confirmed abnormal meiotic behavior existed in hybrids. The hybrids couldn't produce boll-set. Simple sequence repeat results found that besides the fragments inherited from the two parents, some novel bands were amplified in hybrids, indicating that potential mutations and chromosomal recombination occurred between parental genomes during hybridization. These results may provide some novel insights in speciation, genome interaction, and evolution of the tetraploid cotton species.

Introduction

Cotton has important economic values in the world. It has been widely used in different fields, such as in the textile industry, in the production of oil and plant protein, and especially in taxonomic and evolutionary studies [1]. The cotton genus *Gossypium* includes 51 worldwide-distributed species, of which 46 are diploid ($2n = 2x = 26$) and the other five are tetraploid ($2n = 4x = 52$) [2]. Based on chromosomal pairing relationships and geographical distribution, the diploid species can be divided into eight subgenomes and designated as A to G and K [3]. Over the decades, various kinds of interspecific hybrids have been successfully established,

such as *G. arboreum* × *G. raimondii* [4], *G. hirsutum* × *G. sturtianum* [5], *G. arboreum* × *G. bickii* [6], *G. hirsutum* × *G. klotzschianum* [7], *G. hirsutum* × *G. raimondii* (Benbouza et al, 2010), *G. hirsutum* × *G. trilobum* (D9) [8], *G. arboreum* × *G. anomalum* [9], *G. hirsutum* × *G. anomalum* [10], *G. herbaceum* × *G. australe* (G2) [11], etc. Currently, it is well accepted that hybridization plays a major role in cotton plant breeding due to the increase of the genetic diversity available and the production of new genotypes [12].

Gossypium allopolyploids, which are currently responsible for 95% of world cotton production and have high quality levels, are the result of the hybridization between two diploid species (A-genome and D-genome) approximately 1–2 million years ago [13, 14]. The diploid species *G. herbaceum* (A1) and *G. raimondii* (D5) are the progenitors of allotetraploid cotton [13]. They are highly similar to the relative tetraploid genomes at the molecular level and the EST-SSR sequences [15–17], and are important to study the evolution of cultivated cotton and fiber related genes. However, until now, hybrids between *G. herbaceum* and *G. raimondii* haven't been reported.

In the present study, artificial interspecific hybrids (A₁D₅) were produced by crossing *G. herbaceum* (Genome A₁A₁) with *G. raimondii* (Genome D₅D₅). The main goal of this research was to investigate the breeding program of this new germplasm, to evaluate its morphology and cytology, as well as to identify its molecular characteristics. These results may provide novel insights in speciation, genome interaction, and chromosomal recombination occurred between parental genomes during hybridization.

Materials and Methods

Plant materials

The cultivated diploid cotton cultivar *G. herbaceum* 'Redstar' was used as the female parent to cross with a wild diploid cotton accession *G. raimondii* as the male parent. *G. herbaceum* cultivar was an inbreed line continuously self-pollinated for more than 20 generations. The pollen of *G. raimondii* was collected from the China National Wild Cotton Nursery in Sanya, China.

Interspecific hybridization

Hybridization was conducted in the winter season of 2012–2013 in Sanya, China. The flowers for the female parent, which were going to bloom in the next day, were emasculated and stigmas were covered with waxed tubes to avoid cross-pollination. During the blooming period (8:00 AM to 12:00 PM) of the next morning, the emasculated female flowers were pollinated three times with *G. raimondii* pollen. The crossed seeds were harvested from the female plants and planted in nutrition pots. Two week old-seedlings were transplanted into the field at Sanya on May 15, 2014.

Morphological analyses

Fully expanded leaves from the same position on the parents and the hybrid plants were characterized, including the shape, size, presence of pubescence and gland color. During flower development, the expanded flower morphological traits of the putative hybrid plants were compared with those of their parents, including bracteoles, calyx, corolla, stigma, androecium, pollen and capsule.

Cytogenetic observation

Anthers at different development stages were collected from immature flower buds of the hybrid plants and fixed in freshly prepared Carnoy's fluid (ethanol: acetic acid = 3:1 v/v) [11]

immediately. After 24 hours, the fixed anthers were transferred to 70% ethanol and stored at 4°C for cytogenetic observation. The fixed anthers were then stained on slides using Carbol fuchsin solution for microscopic evaluation. Photographs were taken from freshly prepared slides using an Olympus BX60 microscope with automatic camera. Meiosis was studied using a minimum of 500 pollen mother cells (PMCs).

Molecular identification

Genomic DNA from three hybrid plants and their parental accessions were extracted as previously described with minor modifications [18]. Briefly, DNA was extracted from young leaves using CTAB method and quantified using agarose gel electrophoresis. DNA amplification was carried out in a total volume of 20 μ L containing 2 μ L 10 \times buffer, 1.6 μ L MgCl₂ (25 mM), 0.2 μ L dNTPs (10 mM), 6 μ L template DNA (50 ng / μ L), 2 μ L forward and reverse primers (2.5 μ M), 0.2 μ L Taq polymerase (5 U/ μ L, Sangon), and 8 μ L ddH₂O. Nine pairs of simple sequence repeat (SSR) primers were used for amplification (Table 1). PCR amplification was performed in a DNA Mastercycler (Eppendorf, Germany), using an initial denaturing at 94°C for 4 minutes; followed by 35 cycles of pre-denaturation at 94°C for 3 minutes; denaturation at 94°C for 50 seconds; annealing at 58°C for 50 seconds; elongation at 72°C for 2 minutes; and a final extension at 72°C for 10 minutes. Amplified fragments (20 μ L) were separated by PAGE and resolved by silver staining.

Results

Morphological characteristics of AD interspecific hybrid

To understand the morphological differences between AD interspecific hybrid and parents, more than 500 crossed flowers were investigated. Among them, 11 hybrid bolls with 1–3 seeds per capsule were harvested. Among 27 hybrid seeds obtained, 24 were found to be empty and failed to germinate. Three well-developed hybrid seeds germinated and eventually developed into vigorous hybrid plants. The hybrid plants exhibited prolific growth with extensive monopodial branching, which was similar to *G. herbaceum* and *G. raimondii* (Fig 1). The plant height of *G. herbaceum* varied from 50 cm to 70 cm and *G. raimondii* could reach a height of 5.0 m. Meanwhile, the main stem diameters of *G. herbaceum* and *G. raimondii* were 1.5 cm and 5.0 cm respectively. The average height of hybrids was 3.0 m and the average diameter of their main stems was 3.0 cm. Both parameters were in-between two parents. Flower buds of the hybrids appeared at 45 d after planting and they produced flowers profusely and sequentially. *G. herbaceum* can flower in a whole year in Sanya, China. However, the flowering time of hybrids was from November in the first year to May in the second year, which was similar to *G. raimondii*. In addition, many morphological characteristics of the hybrids were also intermediate between *G. herbaceum* and *G. raimondii*, such as the size and shape of leaves (Fig 2A), flowers (Fig 2B), petals, bracts, calyxes, the number of acuminate lobes and the length of androecium (Fig 2C). The color of gland, anther, pollen and corolla in hybrids was yellow, which was similar to that in *G. herbaceum* (Figs 2C and 3). Bracteoles united at base in hybrids were also associated with *G. herbaceum* (Fig 2C). However, the color of staminal column and filaments in hybrids was deep red, which was associated with *G. raimondii* (Figs 2C, 3C and 3D). Meanwhile, anthers and the number of filaments in hybrids were distinct from those in the parents whose filaments were wide and flatted with clustering of anthers (Figs 2C, 3C and 3D). Hybrids showed no boll-set. The main morphological characters of *G. herbaceum*, *G. raimondii*, and the F₁ hybrids are summarized in Table 2.

Table 1. Sequence information of SSR polymorphism primers (from http://www.cottonmarker.org/cgi-bin/cmd_search_marker.cgi).

Primer number	Forword sequence (5'-3')	Reverse sequence (5'-3')
BNL4108	TCCACCATTCCCGTAAATGT	TGGCCAAGTCATTAGGCTTT
BNL4053	TGAAGGCTTTGAAGCAAACA	AAGCAAGCACCAAGTTAGCC
NAU2026	GAATCTCGAAAACCCCATCT	ATTTGGAAGCGAAGTACCAG
NAU1355	ATCTGTTTACGCCACTCTCC	CCAGCCTTTGACATTTTTTCT
NAU1169	GGGTAGTAGCTTTTATGATAGGG	CCATTCTTCCCCTAATCTCT
NAU1157	GAGTTTGGTTCTGGGTTGAG	GATCCTTTTCATCTCCTCCA
NAU1052	CGCAGATAAAGGATGGATTT	AGAGCTGGAGGACATAACAAA
NAU1042	CATGCAAATCCATGCTAGAG	GGTTTCTTTGGTGGTGAAC
NAU1164	CCAACGCTAATTCTACCTCCT	GCGGGTAATTGTAGTACATGC

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Cytogenetic observation

To further investigate the hybrid specialty at the cytogenetic level, meiosis in hybrids was observed. Some meiotic stages of the hybrids exhibited normal behavior, including normal dyad of PMC (Fig 4A), normal tetrad of PMC, and normal pollen grains (Fig 5E). But abnormal meiosis behavior in PMC from hybrids also existed, such as unequal separation of dyad (Fig 4B), the occurrence of triad partly from non-synchronized separation of dyad (Fig 4C), unequal division with abnormal unbalanced micronucleus formation (Fig 4D), and various abnormal polyads (Fig 4E) in telophase II. These results are due to non-synchronous chromosome separation at anaphase I in hybrid meiosis. Of 500 multispores, there were 104 dyads (20.80%), 44 triads (8.80%), 197 tetrads (39.40%) and 155 other polyads (31.00%) respectively (Table 3). Among the 197 tetrads, 120 were normal tetrads (60.91%) and 77 were abnormal tetrads (39.09%). Abnormal tetrads could not form into normal pollen grains, so various abnormal pollen grains were observed, including big oval-shaped pollen, small under-developed

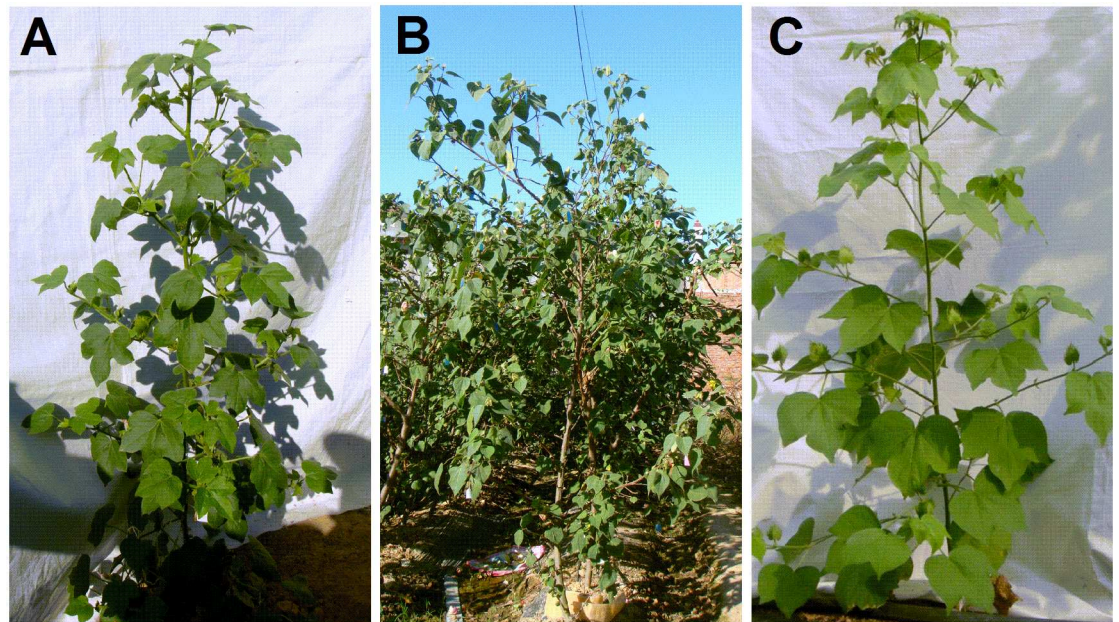


Fig 1. The images of *Gossypium herbaceum* (A), *G. raimondii* (B), *Gossypium herbaceum* × *G. raimondii* F1 hybrid (C).

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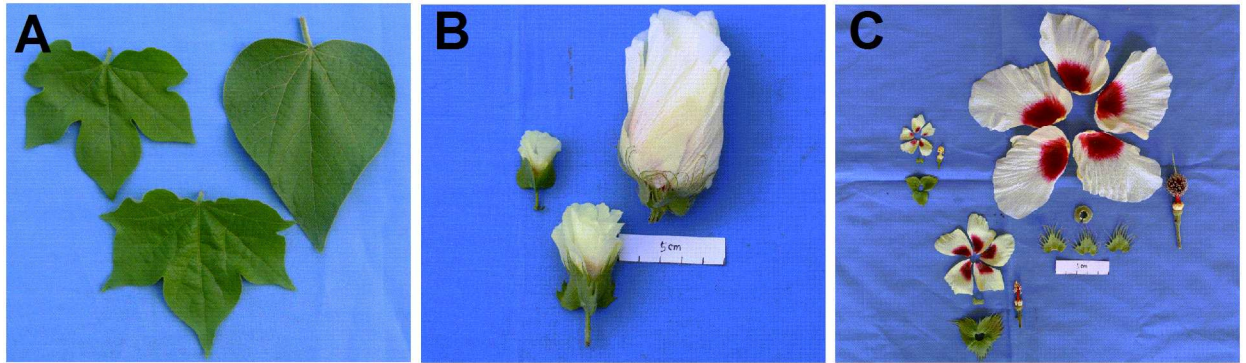


Fig 2. Morphological characteristics of *G. herbaceum* (upper, left), *G. raimondii* (upper, right) and *Gossypium herbaceum* × *G. raimondii* F1 hybrid (lower). A) Leaf. B) Flower. C) Petals, bracts, calyxes, staminal column and filaments.

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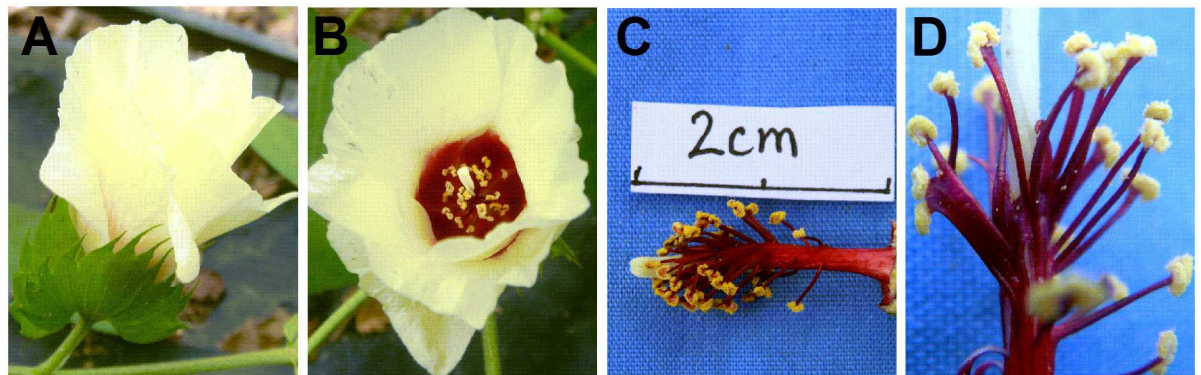


Fig 3. Flower characteristics of *Gossypium herbaceum* × *G. raimondii* F1 hybrid. A-B) Flower. C-D) Staminal column and filaments.

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Table 2. Comparison of main characters of *G. herbaceum*, *G. raimondii*, and *G. herbaceum* × *G. raimondii*.

Organs	<i>G. herbaceum</i>	<i>G. herbaceum</i> × <i>G. raimondii</i>	<i>G. raimondii</i>
Leaf	22–95 mm length, 25–100 mm width; 5 lobed	20–110 mm length, 15–125 mm width; entire or 2–5 lobed	12–170 mm length, 12–150 mm width; entire
Gland color	Yellow	Yellow	Cream
Bracteoles	15 mm length, 14 mm width; 7–8 ebeate lobes, 1–6 mm length, united at base	26 mm length, 25 mm width; 10–12 acuminate lobes, 2–11 mm length, united at base	40 mm length, 35 mm width; 15–16 acuminate lobes, 5–20 mm length, free at base
Calyx	Tube, 2–3 mm length; 7–8 lobes, 0.5–1.0 mm length	Tube, 3–5 mm length; 5 lobes, 1–2 mm length	Tube, 5–6 mm length; united
Corolla	Yellow; small petal spot, deep red	Yellow; large petal spot, deep red	Cream; very large petal spot, deep red
Stigma	Light yellow; 3–5 mm length	Light yellow; 4–7 mm length	Light yellow; 3–5 cm length
Androecium	Light yellow staminal column and filaments; yellow anther cases	Deep red staminal column and filaments; yellow anther cases	Deep red staminal column and filaments; red anther cases
Pollen	Yellow	Yellow	Cream
Capsule	3 locules; 1–6 seeds per locule	No boll setting	3 locules; 2–3 seeds per locule
Pubescence	Puberulent	Canescent	Canescent

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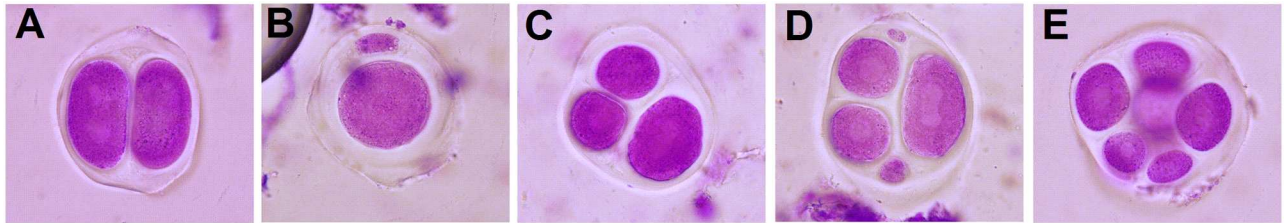


Fig 4. The normal and abnormal meiosis behavior of *Gossypium herbaceum* × *G. raimondii* F1 hybrid. A) Normal dyad. B) Unequal separation of dyad. C) Non-synchronized division of dyad in meiosis II. D) Unequal division with various abnormal micronuclei. E) Abnormal polyads.

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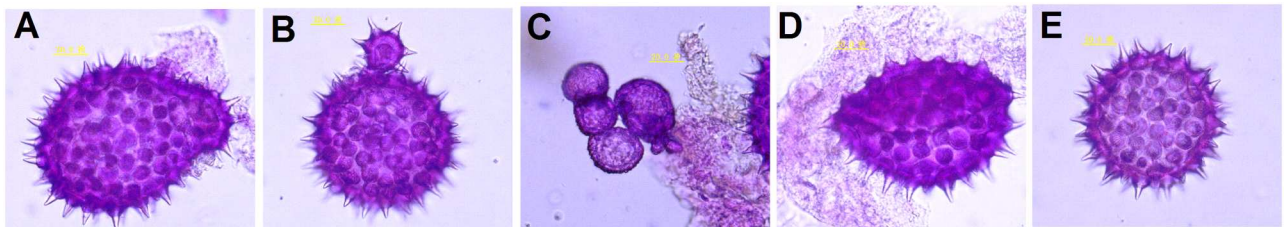


Fig 5. Various abnormal pollen grains after meiosis of *Gossypium herbaceum* × *G. raimondii* F1 hybrid. A) Big oval-shaped pollen. B) Small under-developed pollen. C) Deformed pollen. D) Cracked pollen. E) Normal pollen.

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pollen, deformed pollen, cracked pollen and so on (Fig 5). Among the 500 selected pollen grains, only 292 pollen grains (58.40%) appeared to be normal.

Molecular analysis

To better understand the differences between the hybrid and parents on a molecular level, SSR analysis of the three hybrids and the two parents was performed using nine SSR primer pairs (Table 1) and all yielded microsatellite products. Among these products, the bands amplified by primer BNL4053 in the three hybrids exhibited the complementary contribution from the two parents (Fig 6A). Other than the bands inherited from the two parents, novel bands were produced in hybrid plants using primers NAU2026, NAU1157 and NAU1042. Novel bands were amplified in all three hybrids by primer NAU2026 (Fig 6B, arrow showing). Furthermore, two novel bands in hybrid 3 were produced by primers NAU1157 (Fig 6C, arrow showing) and NAU1164 (Fig 6D, arrow showing) respectively, indicating that there were mutations and chromosomal recombination occurred between parental genomes during hybridization.

Discussion

Polyploidy is a prominent process in plant evolution, especially among angiosperm species [19–22]. All angiosperms are believed to have experienced at least one round of whole-genome duplication during their evolution [23]. The reunion of genomes through hybridization and

Table 3. Number of multispores in telophase II of AD hybrid meiosis.

Shape	Dyad	Triad	Tetrad	Polyad	Total
No	104	44	197	155	500
%	20.80	8.80	39.40	31.00	100

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allopolyploidy may account for 2–4% of speciation events in flowering plants and 7% in ferns [24]. Many of the world's leading crops are polyploids, including genus *Gossypium* [25]. The genus *Gossypium* has been widely used for investigating emergent consequences of polyploidy. *Gossypium* allopolyploids are the result of hybridization between two diploid species (A-genome and D-genome), which occurred approximately 1–2 million years ago [13, 14]. Due to geographical isolation and genetic differentiation, the different tetraploid species are evolved [26, 27]. Compared with the cotton produced by parallel improvement of A genome diploid cottons, domestication and breeding of AD tetraploid cottons have higher fiber yield and quality levels. This comparison implied that the merger of two genomes with different evolutionary histories in a common nucleus can offer unique avenues for phenotypic response to selection [28]. It has been demonstrated that the diploid species *G. herbaceum* (A1) and *G. raimondii* (D5) are the progenitors of allotetraploid cotton [14]. In the present study, the new interspecific hybrids (A₁D₅) between *G. herbaceum* and *G. raimondii* was first produced (Fig 1), which provided the novel insights of genome interaction and speciation as well as its potential applications.

Phenotypic variations have been found in the genome-wide changes resulting from hybridization. The most common phenotypes of AD hybrids were intermediates between the two parents with some other variations [29–31]. The changes in the morphology characteristics of the new AD interspecific hybrid in the present study are consistent with the above reports. Most of the morphological characteristics of the hybrids were found to be intermediate between *G. herbaceum* and *G. raimondii*. However, the color of glands, anther cases, pollen and corolla, and the state of bracteoles in hybrids were associated with the *G. herbaceum*. The color of the staminal columns and filaments in hybrids was associated with *G. raimondii* (Figs 2 and 3, Table 2). It has been reported that the newly formed hybrids may be inviable or sterile [32]. In this study, the AD hybrid between *G. herbaceum* and *G. raimondii* was also sterile, which supported the above conclusion. Meiosis observation showed that various abnormal meiotic behaviors in PMC and abnormal pollen grains existed in the hybrids (Figs 4 and 5, Table 3), which may be involved in the sterilization of the hybrids.

The phenotypic variation of the hybrids may be associated with a variety of factors, including novel gene combinations, chromosomal rearrangements, etc. [30, 33–39]. DNA molecular markers have been widely used in genetic analyses, breeding and investigations of genetic diversity [26]. Since it has been discovered that microsatellites markers are evenly distributed along chromosomes in cotton, SSRs have been widely used to monitor the introgression of alien chromosome fragments in interspecific hybrids [1, 40]. The transcriptional phenotypes of first generation hybrids should predominantly reflect the basic interaction of parental genomes and their endogenous regulatory factors [41]. Moreover, when A and D genomes combined into a single nucleus, they shared gene regulatory factors, which led to novel regulatory processes and changes in regulatory networks. These novel interactions were considered as a genome shock that accompanies polyploidization and might provide a new source of genetic variation [35, 42, 43]. Furthermore, the novel mutational bands have been amplified in hybridization of *Brassica oleracea* var. *alboglabra* and *B. rapa* var. *purpurea*, suggesting interspecific hybridization may play an important role in the fast evolution of vegetable crops [44]. Our present study also observed the novel bands in the hybrids (Fig 6B, 6C and 6D) from SSR analyses, implying that potential chromosomal combination and mutations also occurred during AD interspecific hybridization. Our findings strongly supported the previous hypothesis that interspecific hybridization is an important driving force to develop new mutants and novel phenotypes during plant evolution [45–47]. Furthermore, considering the difficulty of interspecific hybridization to get AD hybrids in this study, we inferred that there might be high-level genome-wide mutations and/or incompatibility

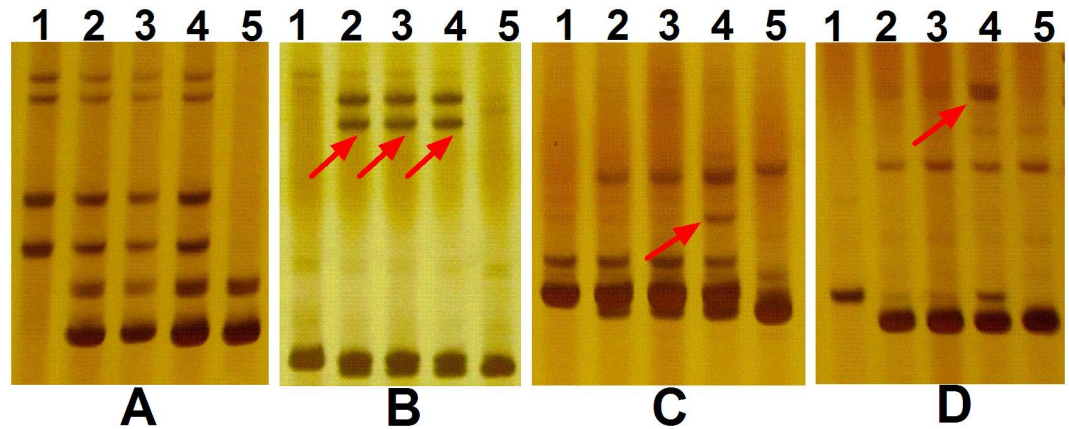


Fig 6. Amplification results for three *Gossypium herbaceum* × *G. raimondii* F1 hybrids and two parents using 4 representative SSR primer pairs. A) BNL4053. B) NAU2026. C) NAU1157. D) NAU1164 respectively. 1-5: *G. herbaceum*, hybrid plant 1, hybrid plant 2, hybrid plant 3, and *G. raimondii*, respectively. The novel bands produced in hybrid plants were indicated by arrows.

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between interacting chromosomes during hybridization to cause the high rate of reproductive failure of hybrids.

In conclusion, artificial interspecific hybrids (A_1D_5) were first produced in the present study by crossing *G. herbaceum* with *G. raimondii*. The breeding program of this new germplasm, its morphological and cytological characteristics, and their molecular identification were analyzed. The novel bands amplified in hybrids indicated the chromosomal combination and mutations occurring in AD interspecific hybridization. These results shed new light on speciation, genome interaction, and evolution of the tetraploid cotton species.

Supporting Information

S1 Fig. Immatured boll. Setting boll on the mutant branch after chromosome doubling by colchicine treatment of this new synthetic hybrid (A_1D_5). (TIF)

S2 Fig. Matured boll. Matured empty boll without developed seeds on the mutant branch after chromosome doubling by colchicine treatment of this new synthetic hybrid (A_1D_5). (TIF)

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Author Contributions

Conceptualization: YW SZ.

Data curation: DC YW.

Formal analysis: LZ LL.

Funding acquisition: YW DC SZ.

Investigation: LL LZ.

Methodology: DC YW.

Project administration: SZ YW.

Resources: SZ.

Supervision: SZ.

Validation: DC LZ.

Writing – original draft: YW.

Writing – review & editing: YW SZ.

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