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Cytogenetic and flow cytometric analysis of new allotetraploid forms derived from wild cotton species with A1 and G genomes

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Abstract

Wild Australian and Afro-Asian cotton species are characterized by their natural resistance to diseases and pests, serving as valuable genetic resources for the introgression of these beneficial traits into cultivated cotton varieties. This study presents the results of research aimed at determining the ploidy level of novel allopolyploid hybrid forms derived from wild Australian (*Gossypium nelsonii*) and Afro-Asian (*G. herbaceum*) cotton species using interspecific hybridization and experimental polyploidy methods. Cytogenetic analyses demonstrated that the interspecific F₁ hybrids possess a diploid chromosome set (2n=2x=26), whereas the synthetic F₁C hybrids developed via experimental polyploidy exhibited a multiplied chromosome set (2n=4x=52). Furthermore, flow cytometry analysis conclusively confirmed this chromosome doubling, showing that the nuclear DNA content doubled from an average value of - 12.8 in the diploid parental forms to 25.492 in the F₁C allotetraploids. The study evaluated the interspecific crossability of species belonging to different genomic groups and successfully generated genetically stable synthetic allotetraploids. This demonstrates the feasibility of utilizing wild diploid species in genetic and breeding research.

Keywords: Cotton, *Gossypium* L., Hybridization, Allopolyploidy, Cytogenetics, Chromosome, Meiosis, Mitosis.

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Introduction

One of the primary obstacles to improving cotton productivity is the narrow genetic base of existing cotton cultivars. In the breeding processes of agricultural crops, including cotton, utilizing closely related species and their wild relatives is considered a promising approach to enriching genetic diversity, particularly in enhancing resistance to biotic and abiotic stresses (1). Genome analysis of wild cotton species provides essential data for expanding the genetic base of existing varieties and serves to ensure variety stability by increasing biodiversity (1). Due to their high potential as sources of beneficial genes, wild species are being extensively utilized in breeding programs to fundamentally improve current cultivars (2). The genus *Gossypium* L. possesses rich genetic diversity and

encompasses numerous species of significant economic value. This genus is also of paramount importance for fundamental research in plant taxonomy, polyploidy, phylogeny, cytogenetics, and genomics (3). The diversity of wild cotton species represents an extensive and critical genetic resource for the breeding process. Currently, various taxa of the *Gossypium* L. genus are distributed globally; specifically, the A, B, E, and F genomes are prevalent across the Asian and African continents, the C, G, and K genomes are indigenous to Australia, and the D and AD genomes are widespread throughout the Americas (4).

Genetic studies on *Gossypium australe*, a species belonging to the Australian flora, have been less extensive compared to other cotton species (5,6,7). Nevertheless, *G. australe* remains a vital resource for modern cotton genomics. Due to

its exceptionally high resistance to *Verticillium* wilt (caused by the fungus *Verticillium dahliae* Kleb.), this species has frequently been selected as a primary subject for genetic research (8,9,10). According to researchers, the implementation of chromosome introgression and translocation from wild species, as well as the development of chromosome substitution lines involving *G. hirsutum*, holds significant scientific importance for enhancing the wilt resistance of medium-staple cotton cultivars (5, 11).

Several scientists have classified diploid cotton species into distinct genome groups (12, 13, 14, 15). Furthermore, significant progress has been made by numerous researchers in developing synthetic hybrids involving Australian (e.g., *G. australe* F. Muell., *G. nelsonii* Fryx., *G. bickii* Prokh.) and Afro-Asian wild species (*G. herbaceum* L.), as well as in evaluating their economically valuable traits (5, 16, 17, 18, 19, 20, 21).

In Uzbekistan, extensive research is also being conducted on interspecific hybridization involving wild cotton species with diverse genomes, the creation of synthetic donors, and the study of trait inheritance patterns (22, 23). The contributions of scientists such as I. Abdurakhmanov and his colleagues (24, 25), as well as F.N. Kushanov (26), are invaluable in assessing the genetic diversity of wild and cultivated species using molecular genetic methods. However, despite several studies on interspecific hybridization in recent years, the successful development of allotetraploid (A1A1G3G3) synthetic donors involving *G. herbaceum* and *G. nelsonii* species, along with their genetic analysis, remains insufficiently explored. Therefore, determining the ploidy levels and conducting cytogenetic analysis of new allopolyploid forms obtained through interspecific hybridization is one of the primary objectives of this research to evaluate their genetic stability.

Materials and Methods

The research was conducted at the Plant Genome Research laboratory of the Institute of Genetics and Plant Experimental Biology, Academy of Sciences of the Republic of Uzbekistan. The study focused on hybrids derived from the Australian wild cotton species *G. nelsonii* Fryx. and Afro-Asian varietal diversities (*G. herbaceum* subsp. *frutescens*, subsp. *euherbaceum* A-256). Diploids obtained through interspecific hybridization and allopolyploid hybrids generated via experimental polyploidy methods were examined, followed by cytogenetic and flow cytometric analyses. Furthermore, the research data were statistically analyzed according to the methods of Dospekhov (27). Phenological observations were carried out throughout the vegetation period. Considering the short-day requirements of wild diploid cotton forms, the research samples were grown in “Wagner” pots placed within specialized photoperiodic chambers.

Seed Germination

Due to the small size and stony seed coat (testa), the micropylar region of the seeds was slightly scarified. The seeds were then germinated in Petri dishes within a thermostat maintained at a temperature of 30–32°C.

The plant material used in this study comprised two wild

diploid cotton species, their initial interspecific hybrids, and the resulting synthetic allotetraploids. The parental genotypes consisted of the Afro-Asian species *Gossypium herbaceum* L. ($2n = 2x = 26$, A1A1 genome) and the wild Australian species *Gossypium nelsonii* Fryx. ($2n = 2x = 26$, GG genome). The primary diploid F_1 hybrids ($2n = 2x = 26$, A1G genome) were generated through controlled interspecific hybridization (*G. herbaceum* × *G. nelsonii*). To restore fertility and induce chromosome doubling, the F_1 materials were subjected to colchicine treatment, successfully yielding the synthetic F_1C allotetraploids ($2n = 4x = 52$, AAGG genome). To ensure the statistical reliability and reproducibility of the cytogenetic and flow cytometry analyses, a strictly defined number of biological replicates was evaluated. Specifically, we analyzed 15 individual plants of *G. herbaceum*, 15 plants of *G. nelsonii*, 15 diploid F_1 hybrids, and 15 colchicine-induced F_1C allotetraploids. All evaluated plants were cultivated under uniform greenhouse conditions at the Institute of Genetics and Plant Experimental Biology.

Experimental Polyploidy Method

To induce chromosome doubling, a portion of the germinated seeds was treated with a 0.02% colchicine alkaloid solution for 20 hours at room temperature under dark conditions.

2.3. Sowing and Cultivation Both the germinated control seeds and those treated with colchicine were sown in paper pots filled with a 1:1 soil-to-sand mixture. Once the seedlings developed 2–3 true leaves, they were transplanted into “Wagner” pots for further growth and phenological observation.

Cytogenetic Analysis

Comprehensive cytogenetic analyses were conducted to determine the ploidy levels of the allopolyploid hybrids. Initially, samples were collected from the root meristems of germinated seeds to study mitotic activity. The tissues underwent hydrolysis with a 30% aqueous H_2SO_4 solution at 35°C for 15 minutes and were subsequently stained with aceto-orcein for 4 - 5 minutes. Temporary slides were examined under a light microscope, and samples with clearly visible chromosome sets were documented via microphotography.

Along with the analysis of somatic cells, the meiotic division process in generative organs and potential aberrations in microspore formation were investigated. For this purpose, flower buds (2 - 4 mm) were collected and fixed in an aceto-ethanol solution (3:7 ratio) at room temperature. The fixed pollen mother cells were stained with acetocarmine to prepare squash slides, which were then analyzed under both low (10×) and high (40×) power microscope objectives. The obtained data were statistically analyzed according to the methodology of Pausheva (28).

Flow Cytometry Method

In the subsequent stage, flow cytometry was employed to evaluate the relative nuclear DNA content of the samples using a Ploidy Analyser (Sysmex Partec GmbH, Görlitz, Germany) equipped with CyView software. For the analysis, a nuclear suspension was prepared from fresh leaf tissues. Nuclei were isolated by lysing cell fragments from the suspension according to the method of Galán (29). DNA

staining was performed using the fluorochrome propidium iodide (PI) at a concentration of 50 µg/ml, along with RNase enzyme. The incubation period lasted 30 minutes in the dark. The DNA content-based relative fluorescence signal was measured and analyzed. Signal intensity, mean relative fluorescence values (represented as relative channel numbers), coefficients of variation (CV), and peaks were recorded. The relative DNA content (2C, 4C) was evaluated to accurately determine the ploidy levels (2x for diploids, 4x for the AAGG allotetraploids) of each sample, and corresponding histograms were generated. The obtained results were formatted for statistical analysis according to the methodology of Doležel J. (30).

For each genotype, a minimum of three biological replicates (n=3) were analyzed to ensure the statistical reliability and reproducibility of the results.

Results

It is well-documented in the literature that interspecific hybrids often exhibit partial or complete sterility due to meiotic segregation disturbances; therefore, applying experimental polyploidy methods is instrumental in restoring fertility and ensuring research efficiency. Chemical mutagens such as colchicine, colchamin, oryzalin, and trifluralin are widely utilized to generate synthetic polyploids. These substances act as antimitotic agents, leading to the disruption of mitotic processes within cells (31). In the present study, a portion of the intergenomic F₁ hybrids was treated with colchicine (C22H25NO6), resulting in the doubling of their chromosome sets and the successful restoration of fertility (Table 1).

Table 1. Interspecific F₁C (A1 × G) synthetic hybrids

No.	Samples	Chromosome set (number)
F₁ diploid hybrids		
1	subsp. <i>frutescens</i> × <i>G. nelsonii</i>	2n=2×=26
2	subsp. <i>euherbaceum</i> A-256 × <i>G. nelsonii</i>	2n=2×=26
F₁C allotetraploid hybrids		
3	subsp. <i>frutescens</i> × <i>G. nelsonii</i>	2n=4×=52
4	subsp. <i>euherbaceum</i> A-256 × <i>G. nelsonii</i>	2n=4×=52

The research findings support the hypothesis that the hybridization of distantly related species is a crucial factor in plant evolution, leading to the emergence of new mutations and, consequently, the formation of new species. By doubling the chromosome sets of F₁ hybrids obtained through the crossing of *G. nelsonii* with *G. herbaceum* L. infraspecific diversities - specifically the cultivated tropical form subsp. *frutescens* and the cultivar “A-256” (subsp. *euherbaceum*) - new genotypes, namely allotetraploid F₁C hybrids, were successfully developed. As a result, the fertility of the previously sterile hybrids was restored. In subsequent studies, hybridization between these allopolyploid hybrids and local cultivars was conducted, yielding positive results.

The F₁ and F₁C hybrids were morphologically compared with their parental genotypes. The investigation revealed a wide amplitude in plant height and the emergence of a new flower color (dark pink) that was not present in the parental forms. Although the F₁ diploid and F₁C amphidiploid plants showed minimal morphological differences, the F₁C plants were distinguished by larger and thicker leaves, fertile pollen, and sturdy, erect stems. All other morphological traits were observed to be identical.

Cytogenetic analysis is considered one of the most critical methods in intergenomic hybridization research. It enhances the efficiency and accuracy of the study by enabling the determination of ploidy levels, analysis of sporads, pollen viability, and the observation of chromosome conjugation and segregation processes. During our research, mitotic processes were investigated to confirm the doubling of the chromosome sets in the allotetraploid hybrids obtained by crossing representatives of the A1 and G genomes (Figure 1). The results demonstrated that the F₁ (*G. herbaceum* subsp. *frutescens* × *G. nelsonii*) and (*G. herbaceum* subsp. *euherbaceum* × *G. nelsonii*) hybrid plants possess a diploid (2n=2x=26) chromosome set. It was confirmed that the F₁C hybrids are allopolyploid (2n=4x=52) forms.

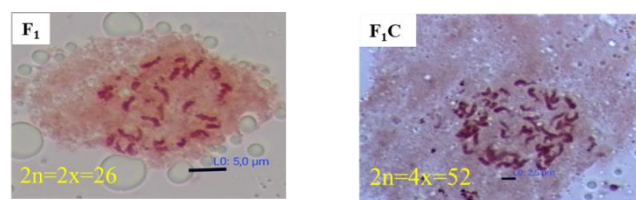


Figure 1. Prophase stage of mitosis in chromosomes. F₁ – hybrid with a diploid set of 26 chromosomes; F₁C – hybrid treated with colchicine (allotetraploid set of 52 chromosomes).

Root tip staining and microscopic observations revealed that the chromosome number in the root tips of *G. herbaceum* and *G. nelsonii* control samples was 26, while the F₁C hybrid possessed 52 chromosomes. These measurements confirmed the tetraploid nature of the F₁C hybrid. Tetrad Analysis. According to the tetrad analysis, it was observed that alongside normal tetrads, aberrations such as micro-nucleated spores and polyads (pentads, hexads, heptads, etc.) occurred in the studied samples (Figure 2 and Table 2).

Normal processes were evident in certain meiotic stages of the hybrids, including normal dyads of the PMC (Pollen Mother Cell), normal tetrads, and normal pollen grains. However, abnormal and irregular meiotic events were also observed in the PMCs of the hybrids. Specifically, these included unequal dyad separation (Figure 2b), the appearance of dyads with five micronuclei due to partially asynchronous dyad division (Figure 2c), and various abnormal polyads in telophase II (Figure 2e).

These results are associated with the slight asynchronous chromosome disjunction during anaphase I of the hybrid meiosis. According to the results of the sporad analysis, the meiotic index in the parental forms ranged from 89.8 ± 1.01% to 95.6 ± 0.9%. These values indicate the stability of meiotic

processes in the parental genotypes. In the F₁ subsp. *frutescens* × *G. nelsonii* hybrid, the proportion of micronucleated sporads was found to be $6.9 \pm 0.8\%$, the proportion of polyads was $2.5 \pm 0.5\%$, and the meiotic index was recorded at $78.6 \pm 1.3\%$. In the F₁ subsp. *euherbaceum* A-256 × *G. nelsonii* hybrid, the proportion of micronucleated sporads was $20.3 \pm 1.7\%$, the proportion of polyads was $5.5 \pm 0.9\%$, resulting in a meiotic index of $57.8 \pm 2.1\%$.

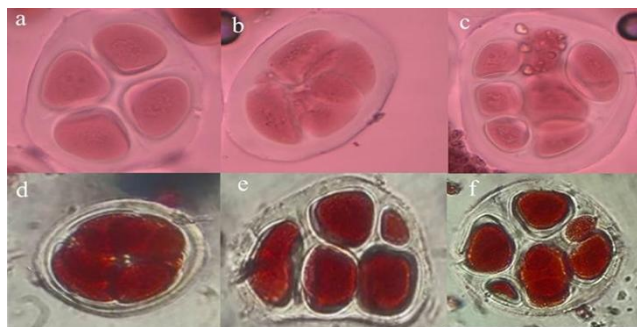


Figure 2. Appearance of normal and micronucleated tetrads in F₁ (*G. herbaceum* subsp. *euherbaceum* × *G. nelsonii*) and F₁C (*G. herbaceum* subsp. *frutescens* × *G. nelsonii*) hybrid forms: a, b, d) normal tetrads; c, e, f) micronucleated sporads.

It was observed that the meiotic index values in the F₁C allotetraploid hybrids (subsp. *frutescens* × *G. nelsonii*) and (subsp. *euherbaceum* A-256 × *G. nelsonii*) were closely similar. Specifically, in the first allotetraploid hybrid sample (subsp. *frutescens* × *G. nelsonii*), the proportion of micronucleated sporads was $37.6 \pm 2.5\%$, the proportion of polyads was $1.8 \pm 0.7\%$, and the meiotic index was $46.8 \pm 2.6\%$. In the F₁C subsp. *euherbaceum* A-256 × *G. nelsonii* allotetraploid hybrid, these indicators were found to be $36.7 \pm 2\%$ and $51.9 \pm 0.7\%$, respectively, with a meiotic index of $47.1 \pm 1.9\%$. The low meiotic index can be attributed to several factors: the chromosomal divergence between distantly related species, which prevents proper homology during metaphase I, causing chromosomes to remain as univalents on the equatorial plane; the subsequent disruption of segregation, leading to the random and irregular distribution of chromosomes to the cell poles; the genomic-cytoplasmic incompatibility between the nuclear and cytoplasmic genomes in the hybrids; and particularly the impact of artificial mutations induced by colchicine treatment. The low pollen viability in complex F₁ hybrids is attributed to the presence of three distinct genomes within their genotype. During the prophase I (zygotene) stage of meiosis, homologous chromosomes undergo pairing (synapsis), leading to the occurrence of crossing over. This leads to the formation of a limited number of normal sporads alongside abnormal ones, such as micronucleated sporads and polyads. It can be inferred that the presence of 13II (13 bivalents) in the pollen mother cells (PMCs) during metaphase I (MI) indicates that this specific stage proceeded normally. However, the low pollen viability in these hybrids suggests that the mitotic stages during gametogenesis did not occur correctly. The decrease in the meiotic index was caused by various meiotic anomalies, including

micronucleated tetrads and polyads. This low value further indicates structural differences in the chromosomal organization of the studied samples.

Flow Cytometry Analysis

In this study, the DNA content (C-value) of plant samples from *G. herbaceum*, *G. nelsonii*, and their resulting diploid and amphidiploid hybrids was evaluated using flow cytometry. The analysis focused on determining the efficiency of the transition from the diploid level to the allotetraploid level in the plants (Figure 3).

Flow cytometry analysis of the *G. herbaceum* and *G. nelsonii* parental samples revealed a dominant G₀/G₁ peak positioned at relative fluorescence channel 200 (mean fluorescence value: 12.776–12.834). This baseline establishes the 2C nuclear DNA content reference for these diploid ($2n = 2x = 26$) species. Similarly, the interspecific F₁ (*G. herbaceum* × *G. nelsonii*) hybrids exhibited a primary peak at the same relative channel, confirming they retained their original diploid ($2n = 2x = 26$) state prior to polyploidization. Following colchicine treatment, the histogram of the synthetic F₁C allotetraploids (AAGG) displayed a distinct shift of the G₀/G₁ peak to relative fluorescence channel 400 (mean value: 25.492). The precise 1:2 ratio of the peak positions (channel ~200 vs. ~400) and the corresponding doubled mean fluorescence values proportionally reflect a twofold increase in nuclear DNA content. This confirms the successful whole-genome duplication and the stable allotetraploid ($2n = 4x = 52$) nature of the F₁C hybrids. The table presents the average DNA values and the coefficient of variation (CV) for the studied samples. The low CV values indicate high precision and the reproducibility of the results across multiple replicates. The presence of a residual diploid peak in the allotetraploid hybrid signifies the existence of cells that did not undergo polyploidization, which is considered a natural component of the experimental process.

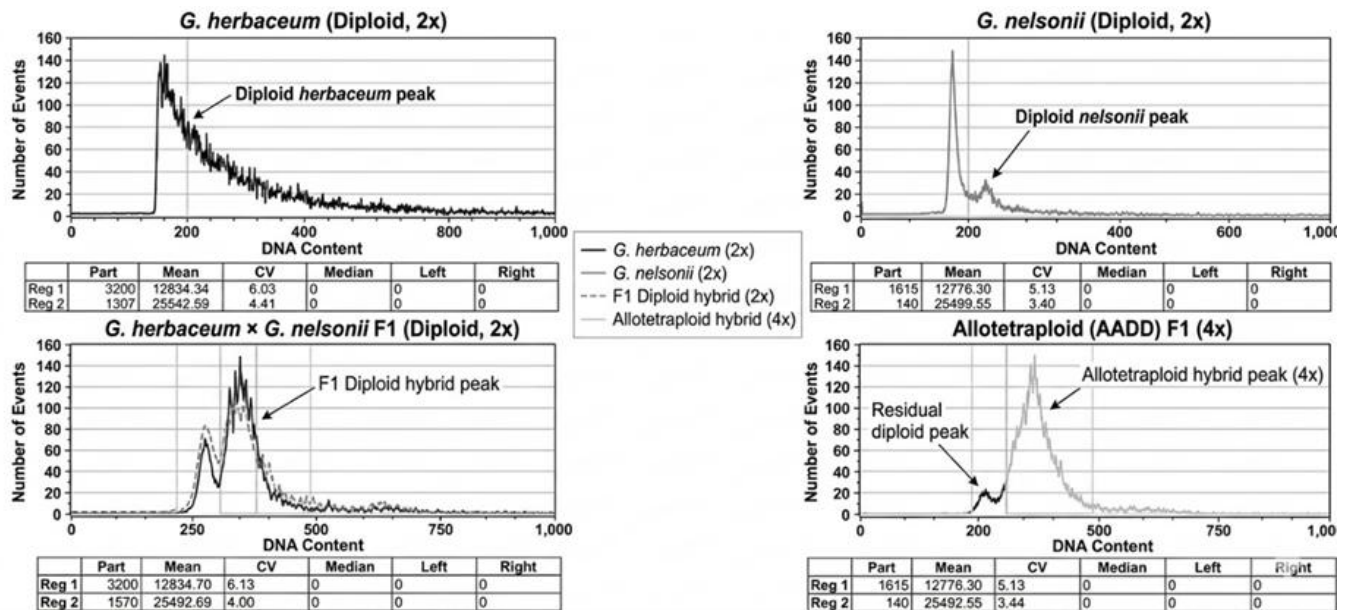
It should be particularly emphasized that the creation of new intergenomic synthetic allotetraploid forms not only enriches the cotton germplasm but also serves as a unique intermediate source for genetic and breeding research aimed at developing new varieties with valuable economic traits. Through “breeding hybridization,” unique genes essential for agriculture - specifically those that enhance resistance to biotic and abiotic stresses - can be introgressed into the genomes of cultivated varieties. Since genetic resources within a single species are often limited, interspecific and, especially, intergenomic hybridization provides the opportunity to achieve significantly higher efficiency in crop improvement.

Discussion

Mechanisms of Hybrid Sterility and Genomic Incompatibility. A critical finding of this study is that despite the meiotic index values in the F₁ diploid hybrids ($57.8\% - 78.6\%$) being relatively close to the parental forms, the plants remained practically sterile. Similar reproductive barriers were reported in the development of synthetic A1A1G2G2 allotetraploids, where diploid hybrids exhibited profound sterility prior to chromosome doubling (6).

Table 2. Tetrad analysis in the studied cotton samples

No	Samples	Total number of sporads	Number of normal tetrads	Meiotic Index, %	Micronucleated sporads, %	Polyads, %	Monads and Dyads, %
Parental forms							
1	subsp. <i>frutescens</i>	787	741	94.2±0.8	—	0.7±0.3	5.1±0.8
2	subsp. <i>euherbaceum</i> A-256	907	814	89.8±1.01	2.65±0.5	2.7±0.5	4.9±0.7
3	<i>G. nelsonii</i>	1003	959	95.6±0.9	—	0.1±0.02	4.3±0.6
F₁ diploid hybrids							
5	subsp. <i>frutescens</i> × <i>G. nelsonii</i>	1011	795	78.6±1.3	6.9±0.8	2.5±0.5	12.0±1.0
6	subsp. <i>euherbaceum</i> A-256 × <i>G. nelsonii</i>	547	316	57.8±2.1	20.3±1.7	5.5 ±0.9	16.4±1.6
F₁C allotetraploid hybrids							
7	subsp. <i>frutescens</i> × <i>G. nelsonii</i>	378	178	47.1±1.9	37.6±2.5	1.8±0.7	13.5±1.8
8	subsp. <i>euherbaceum</i> A-256 × <i>G. nelsonii</i>	380	178	46.8±2.6	36.7±2.5	1.9±0.7	14.6±1.8

Figure 3. Flow cytometric analysis of DNA content and ploidy levels in *Gossypium herbaceum*, *G. nelsonii*, and their hybrids (F₁ and AAGG allotetraploids).

Our embryological observations further explain this phenomenon by revealing a severe reduction in pollen tube penetration into the ovule. This confirms that morphologically formed pollen grains were physiologically non-viable. Unlike previous assumptions that primarily attribute such sterility to meiotic pairing failure (13, 14), our findings indicate that sterility in these distantly related hybrids is intrinsically linked to imbalances during the post-microsporogenesis stage, specifically within the microgametogenesis (mitosis) process. The nucleo-cytoplasmic genomic incompatibility between the A1 and G genomes effectively blocks pollen tube growth, leading to gametophytic sterility even when meiosis concludes successfully.

Restoration of Meiotic Stability through Polyploidization. The application of experimental polyploidy fundamentally altered the reproductive viability of the hybrids, aligning with the morphological and cytological stabilizations observed by Yin et al. (2020) in A1A1G3G3 synthetic forms (20). Experimental polyploidization doubled the chromosome set ($2n=4x=52$), ensuring that each chromosome could pair with its exact homolog in the allotetraploid (F₁C) background. Consequently, our embryological analyses demonstrated a positive shift in pollen tube penetration rates, which directly correlated with stabilized meiotic indices. This proves that polyploidization not only restores the absolute chromosome number - as verified by our flow cytometry

data showing a twofold increase in DNA content - but crucially resolves the microgametogenesis disruptions observed in diploid hybrids. By ensuring genetic and physiological balance, this method effectively bridges the reproductive isolation between Afro-Asian and Australian wild cotton species, paving the way for the introgression of elite disease-resistance traits into cultivated varieties.

Conclusion

This study successfully developed and validated novel synthetic allotetraploids (AAGG) via interspecific hybridization of wild Australian (*Gossypium nelsonii*) and Afro-Asian (*G. herbaceum*) cotton species. The doubling of the chromosome set (from $2n=26$ to $2n=4x=52$) and the exact twofold increase in nuclear DNA content were conclusively verified through robust cytogenetic and flow cytometry analyses. A key breakthrough of this research is the identification of nucleo-cytoplasmic incompatibility at the post-microsporogenesis stage as a primary cause of hybrid sterility. By applying experimental polyploidization, this gametophytic barrier was successfully overcome, restoring meiotic stability and microgametogenesis. Ultimately, these genetically stable F_1C amphidiploids represent a highly valuable “bridge” germplasm. They provide a practical and efficient pathway for introgressing critical traits - such as natural resistance to pests and diseases inherent to wild diploid species - into the narrow genetic base of cultivated commercial cotton varieties.

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Disclaimer: No

Conflicts of Interest: The authors declare no conflict of interest.

Data availability: Available on reasonable request.

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Ethics approval statement: Not applicable

Authors' Contribution Statements: F.U.R. and B.B.O. conceived and designed the research. M.D.K. conducted the cytogenetic and flow cytometry analyses. A.K.T. and S.K.A. analyzed the data and drafted the manuscript. D.K.E. and F.N.K. managed the project. All authors contributed to manuscript revisions and approved the submitted version.

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