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Phenotypic, Cytological and Molecular (AFLP) Analyses of the Cotton Synthetic Allohexaploid Hybrid (*G. hirsutum* × *G. longicalyx*)^2

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Abstract

The wild cotton diploid species (2n = 2x = 26) are important sources of useful traits such as high fiber quality, resistance to biotic and abiotic stresses etc., which can be introgressed into the cultivated tetraploid cotton *Gossypium hirsutum* L (2n = 4x = 52), for its genetic improvement. The African wild diploid species *G. longicalyx* Hutchinson and Lee could be used as donor of the desirable traits of fiber fineness and resistance to reniform nematode. However, hybridization of wild diploid species and cultivated tetraploid cotton encounters a sterility problem of the triploid (2n = 3x = 59), mainly due to ploidy. The restoration of the fertility can be done by creating an allohexaploid (2n = 6x = 78) through the doubling with colchicine of the sterile triploid chromosomes. With this method, a synthetic allohexaploid hybrid (*G. hirsutum* × *G. longicalyx*)^2 has been obtained. This genotype was studied using phenotypic, cytological and molecular (AFLP) analyses in order to confirm its hybridity and its caryotype, and also to verify the expression of the desirable traits coming from *G. longicalyx*. The studied genotype showed a quite good level of pollen fertility (83%), and apart from larger seeds and some minor seedling anomalies, most of its morphological characteristics were intermediate between the two parental species. It had 78 chromosomes, proving its hexaploid status. Molecular analysis revealed 136 AFLP loci in this hexaploid, all from *G. hirsutum* and *G. longicalyx*, demonstrating its hybrid status. In addition, the hexaploid exhibited the useful traits of *G. longicalyx* with regard to its remarkable fiber fineness and its high resistance to the reniform nematode. This allohexaploid hybrid constitutes an interesting agronomic material, which can be used as a bridge for the transfer of useful agro-

nomic traits from wild species to varieties of *G. hirsutum*.

Keywords

Gossypium spp, Hexaploid Hybrid, Chromosomes, AFLP Marker, Plant Breeding

1. Introduction

Cotton, from the genus *Gossypium*, is the most important natural fiber source for the textile industry in the world [1]. The *Gossypium* genus is composed of 53 species among which 7 are tetraploid (2n = 4× = 52) and 46 are diploid (2n = 2x = 26) [2]. Diploid cottons are classified into 8 genome types, denoted A-G and K, based on chromosome pairing relationships [3] [4]. The tetraploid cottons have a genome designated by AD, which resulted from the ancestral allopolyploidization of progenitor A-genome and D-genome diploids about 1-2 million years ago [2] [5].

Two cotton diploids species (*G. arboreumn* L and *G. herbaceum* L) and two tetraploids species (*G. hirsutum* L and *G. barbadense* L) are cultivated for their spinnable fiber [6] [7] [8]. *G. hirsutum*, known as Upland cotton, provides more than 90% of the world's cotton production due to its high yield [8]. The remaining cotton fiber supply is produced from the other three cultivated cottons [9]. Apart from these four species, the other 46 species of *Gossypium* are wild.

Genetic improvement of the main cultivated cotton *G. hirsutum* can be done using wild species as donor of traits of interest [10]. Indeed, wild diploid species are important sources of several desirable genes to improve fiber quality, resistance to diseases and insect pests, or tolerance to abiotic stress of Upland cotton [4] [11]. The African wild species *G. longicalyx* Hutchinson and Lee (F1 genome) represents an interesting source of genes that can potentially be transferred to the main cultivated cotton species. This wild diploid species could be used as donor of the desirable trait of fiber fineness, which is very important to textile industry [4] [12] [13], and also as donor of the resistance to reniform nematode [14] [15].

Technically, the use of wild diploid species to improve cultivated tetraploid cotton faces the problem of the sterility of the triploid (2n = 3x = 59) obtained, mainly due to ploidy. To overcome this problem, a strategy used involves the creation of an allohexaploid hybrid as a bridge genotype. This method begins with the hybridization of *G. hirsutum* with a diploid wild species to obtain a sterile triploid (2n = 3x = 59). The next step consists in doubling the chromosome number of the sterile triploid with colchicine to give a fertile allohexaploid (2n = 6x = 78). Such a synthetic allohexaploid hybrid could subsequently be used to introgress the alien genes through trispecific hybrid or monosomic addition lines [16] [17] [18]. In cotton breeding, the successful use of this method has

been demonstrated by the effective introgression of alien chromosomic material into upland cotton and the expression of the beneficial effects of exotic genes [4] [11] [19] [20].

At the Laboratory of Tropical Agro ecology of Gembloux Agro-Bio Tech (Liège University, Belgium) a synthetic allohexaploid hybrid (*G. hirsutum* × *G. longicalyx*)^2 has been created. As a prelude to the use of this hexaploid in a breeding program, the present study aimed at its phenotypic, cytological and molecular analysis (AFLP markers) in order to confirm its hybridity and check its expression of the desirable traits from *G. longicalyx*.

2. Material and Methods

2.1. Plant Material

Plant material included plants of a synthetic allohexaploid hybrid (*G. hirsutum* x G. *longicalyx*)^2 and its parental species *G. longicalyx* and *G. hirsutum*. These plants were obtained from seeds coming from the collection of the Tropical Agro Ecology Laboratory of Gembloux Agro-Bio Tech (University of Liège, Belgium). In the crossing scheme used to generate the allohexaploid hybrid (*G. hirsutum* × *G. longicalyx*)^2, the tetraploid *G. hirsutum* ($A_1A_1D_1D_1$, 2n = 4× = 52) was crossed with the diploid *G. longicalyx* (F_1F_1, 2n = 2× = 26) to give triploid hybrid ($A_1D_1F_1$, 2n = 3x = 39) seeds. Hybrid seedling plants were then treated with 0.15% colchicine for chromosome doubling and an allohexaploid ($A_1A_1D_1D_1F_1F_1$) with 78 chromosomes was obtained. This putative synthetic allohexaploid hybrid produced flowers and set bolls normally. Its seeds were used to produce the plants that are investigated in the present study. The plants were grown in greenhouse, in 5 liter pots filled with a 3:2:1 (v:v:v) sterile mixture of compost, sand and peat.

2.2. Cytological Analysis

To check the chromosome numbers of the hexaploid and its parental species, mitotic chromosome preparations were carried out using root tips. Fast-growing root tips were collected in 0.04% 8-hydroxyquinoline for 4 hours at room temperature (RT) and fixed for 48 h in a fresh fixative fluid (3:1 ethanol: acetic acid) at 4˚C. After washing in distilled water (2 × 10 min), treating in 0.25 N HCl (10 min), rinsing in distilled water (10 min) and treating in a 0.01M citrate buffer (10 min), root tips were digested in an enzyme solution (5% cellulase Onozuka R-10, 1% pectolyase Y-23 in citrate buffer) at 37˚C for 1 hour. The enzyme mix was removed by rinsing in distilled water for 10 min, and on a clean glass slide a single root tip was spread in one or two drops of fresh fixative (3:1 ethanol:acetic acid) using a fine-pointed forceps. After staining with 4,6-diamino-2-phenylindole/Vectashield, mitotic metaphase plates were visualized and the chromosomes were counted under fluorescent light with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a JVC KY-F 58E camera (JVC, Yokohama, Japan).

2.3. Phenotypic Observations

Morphological observation and plant fertility evaluation

The appearance, shape and size of the hexaploid seeds were observed and compared to those of its parental species and their germination rates were assessed. The test of germination was conducted on 44 seeds of the hexaploid and 30 seeds of each of its parental species. The seeds were placed to germinate in Petri dishes with moist filter paper at 28˚C. The criterion for germination was a radicle length of > 1 mm. The germination rates were calculated as the percentage of seeds that germinated from the total number of seeds placed in the Petri dish.

The morphological observations carried out on the plants concerned: the aspect of seedlings (presence of malformations or not), the shape and the size of the leaves, the number of main stem nodes, the final plant height, the flower aspect and the capsule size.

For pollen fertility evaluation, about 300 pollen grains per plant were analyzed. Pollen grains collected in the morning on the day of anthesis were dipped in a drop of 1.5% acetic-carmine solution on a slide for 30 minutes and were analyzed under a stereomicroscope Nikon Eclipse E800 (Nikon, Tokyo, Japan). Only fully stained and large pollen grains were scored as viable and non-aborted. The quantity of viable pollen was estimated as the percentage of stained pollen.

The self-fertility was assessed by determining the average number of seeds obtained per self-pollinated flower. The cross-fertility was assessed by counting the average number of seeds obtained per cross-pollinated flowers.

Fiber fineness analysis

For fiber fineness analysis, cotton fibers were harvested at full maturity and used for the analysis. The fibers were combed and a tuft of parallel fibers was cut from the seed. Their free points were also cut and the median region was placed on a slide and covered with a cover glass. We let one or two drops of 18% NaOH solution penetrate by capillarity into the fibers. The NaOH solution swells the fibers. The diameter of at least 100 fibers was then measured with the software NIS-Elements BR 2.30 (Nikon, Japan) using the Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a digital JVC KY-F 58E camera (JVC, Yokohama, Japan). The ribbon width was determined by dividing the mean of the diameters measured by the 1.3 Summers coefficient [4] [13] [21]. The data collected were subjected to the analysis of variance (ANOVA) using the software Statistica 7.1 (Stat Soft, France). The least significant difference (LSD) was used to establish differences between means at $P = 0.05$.

Evaluation of resistance to reniform nematode

The resistance to reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira) was assessed following the protocol of [15]. Briefly, 30-days seedlings planted in 20-cm-diameter plastic pots, were inoculated with 6000 eggs of *R. reniformis*. Sixty days after inoculation, the soil was gently removed, the roots carrying the nematode eggsacs were weighed and the reniform nematode eggs were then extracted and

counted according to a NaOCl-Blender-Sieving-Centrifugation-Flotation method. For each plant, the number of eggs per gram root was determined. These numbers were used to assess the relative resistance of each plant compared to the susceptible control *G. hirsutum* by calculating the percentage of eggs per gram root for each plant considering the 100% level for the susceptible control. The scale of relative resistance used contains the following classes: 0 - 10% = highly resistant, 11 - 25% = resistant, 26 - 40% = moderately resistant, 41 - 60% = low susceptible, 61 - 100% = susceptible as control, and above 100% = very susceptible. As this technique for evaluating resistance to reniform nematode was destructive, before their evaluation the plants were grafted onto vigorous seedlings of *G. hirsutum* in order to keep a copy of each of them.

2.4. Molecular Analysis

DNA isolation

Total genomic DNA of *G. hirsutum*, *G. longicalyx* and two synthetic allohexaploid (*G. hirsutum* x *G. longicalyx*)^2 plants were isolated from young fresh leaf tissues following the CTAB method as described by [22].

AFLP Analysis

AFLP was performed by Automated Laser FLuorescence (ALF) analysis. Three AFLP primer combinations were used: E-ACC/M-CAG, E-ACT/M-CTG and E-ACT/M-CAG. AFLP was carried out using the "AFLP Analysis System I / AFLP starter primer kit" (Invitrogen, Belgium) following the protocol proposed by Invitrogen. Briefly, genomic DNA (250 ng) was double digested with EcoR I and Mse I restriction endonucleases. The digested DNA fragments were ligated to EcoR I and Mse I adaptors with T4 DNA ligase to generate template DNA for amplification by PCR. Two consecutive PCR were performed: a pre-selective and selective PCR. In the pre-selective reaction, DNA was amplified using an AFLP pre-amp primer pair complementary to the adaptors and each having one selective nucleotide. Pre-selective PCR amplification was used as template for the selective amplification using AFLP primers, each containing three selective nucleotides. The PCR amplification products were run on 6% denaturing polyacrylamide gel using the ALF-Express (Pharmacia Biotech, Freiburg, Germany), which is an Automated Laser FLuorescence DNA sequencer. The obtained digital image of the profiles was analyzed. The scoring of bands was done as present (1) or absent (0) for AFLP marker loci and data were entered in a binary data matrix as discrete variables.

3. Results and Discussion

3.1. Mitotic Chromosome Analysis

Analysis of the mitotic metaphase plates showed 52 chromosomes for *G. hirsutum*, 26 chromosomes for *G. longicalyx* and 78 chromosomes for the putative (*G. hirsutum* × *G. longicalyx*)^2 hexaploid hybrid (**Figure 1**). This number of 78 chromosomes proves the hexaploid status of the material studied because it is in

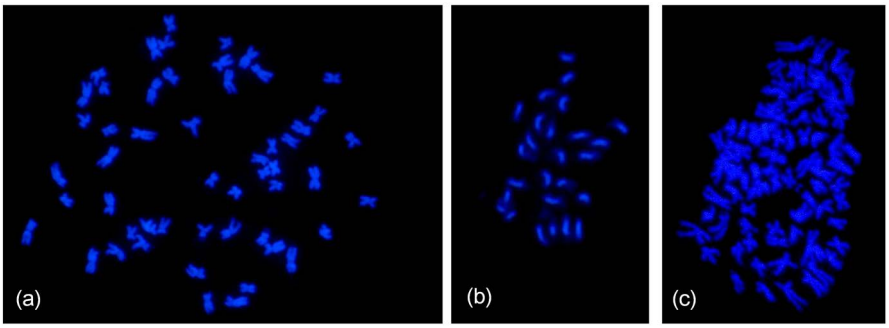

Figure 1. Chromosomal configuration at mitotic metaphase: (a) 52 chromosomes of *G. hirsutum* (×1500); (b) 26 chromosomes of *G. longicalyx* (×1000); (c) 78 chromosomes of the (*G. hirsutum* × *G. longicalyx*)^2 hexaploid hybrid (×1500).

agreement with the number of chromosomes expected for a synthetic hexaploid cotton (2n = 6x = 78) resulting from the doubling of the chromosomes of a triploid issued from the cross between a tetraploid cotton (*G. hirsutum*, 2n = 4x = 52) and a diploid cotton (*G. longicalyx*, 2n = 2x = 26). This result confirms that artificial somatic allopolyploidization can be successfully accomplished *in vivo* through the use of antimitotic reagents, *i.e.* metaphase inhibitors such as colchicine [23] [24]. This reagent causes the depolymerization of the microtubular cytoskeleton in the early phases of metaphase, blocking the separation of chromosomes in mitoses, consequently leading to polyploidization of the cells [25].

3.2. Seed Aspect, Germination Rate and Seedling Abnormalities Analysis

The seeds of the (*G. hirsutum* × *G. longicalyx*)^2 hexaploid hybrid had normal appearance and shape but they were all larger than the seeds of the parental species (**Figure 2**). This result is in accordance with [24] and [26] who reported that polyploids have usually larger seeds than parental species. It is probably a direct consequence of large cell size in polyploids [27], since genome duplication increases cell volume by increasing genome size [28]. This is in line with the expectation that the sizes of seed are a function of cell size, which is larger in polyploids [29].

In the germination test, the seeds of the hexaploid hybrid showed the relative lowest germination rate with 38 germinated seeds on 44 (86.36%) compared to its parental species *G. hirsutum* and *G. longicalyx*, which respectively presented 100% and 96.67% (29 germinated seeds on 30) germination rates. The germination rate gives an estimate of the viability of the seeds. The result obtained suggests a problem of viability of about 13% of the seeds produced by the allohexaploid hybrid. [24] working on synthetic polyploidy in *Hylocereus monacanthus* also reported problems of seed viability. The genome of newly formed polyploid plants usually undergoes extensive genetic and epigenetic changes which can alter gene expression and generate physiological changes that can affect seed viability [28] [30] [31].

(a) (b) (c)

Figure 2. Seeds of the hexaploid hybrid and its parental species: (a) smaller seed of *G. longicalx*; (b) intermediate seeds size of *G. hirsutum*; (c) larger seeds of the hexaploid (*G. hirsutum* × *G. longicalyx*)[2].

Some of the hexaploid seedlings obtained from the 38 germinated seeds, presented some abnormalities at cotyledon leaves stage (**Figure 3**). Three types of abnormalities were observed: i) cotyledonary leaves welded on the petioles (**Figure 3(a)**, 4 seedlings), ii) cotyledonary leaves welded on the entire length of the leaves (petioles and blades) giving the impression of a unique leaf (**Figure 3(b)** and **Figure 3(c)**, 3 seedlings), iii) and seedlings with a normal cotyledonary leaf associated with a progressive necrosis of the other cotyledonary leaf (**Figure 3(d)**, 3 seedlings). This necrosis consisted in a prolonged chlorosis of the cotyledonary leaf. It leaded to the premature death of the concerned leaves but not of the plant. However, [32] reported such an anomaly in polyploid wheat, where it ultimately caused the death of the whole plant in certain wheat hybrids. All abnormalities observed in the hexaploid seedlings were not observed in the parental *G. hirsutum* and *G. longicalyx* species. Out of 38 germinated hexaploid seeds, 10 seedlings showed one or other of the anomalies observed, *i.e.* 26.31% malformed seedlings. According to [33], such abnormalities could result in lethal or semi-lethal condition that imposes a great barrier when trying to transfer desirable traits between species. Actually, after polyploidization the architecture of the cell is modified, the cell must adapt to the new nuclear DNA content and deal with changes in the homology of the chromosomes, gene expression and epigenetics [25]. Changes in gene expression and physiological processes due to genome duplications can generate anomalies that can be deleterious for the development of the polyploid individual [25]. Fortunately, only 26.31% of the hexaploid plants in the present study presented anomalies, which were not necessarily fatal for the plants. Indeed, the malformations observed seemed to be minor and they had no detrimental consequences on the development of the plants except for a seedling with the abnormality of the fully welded cotyledonary leaves. This seedling remained at cotyledon leaves stage for a long time until death.

3.3. Analysis of Morphological Observations of the Plants

The height of the hexaploid plants varied from 152 to 236 cm with an average of

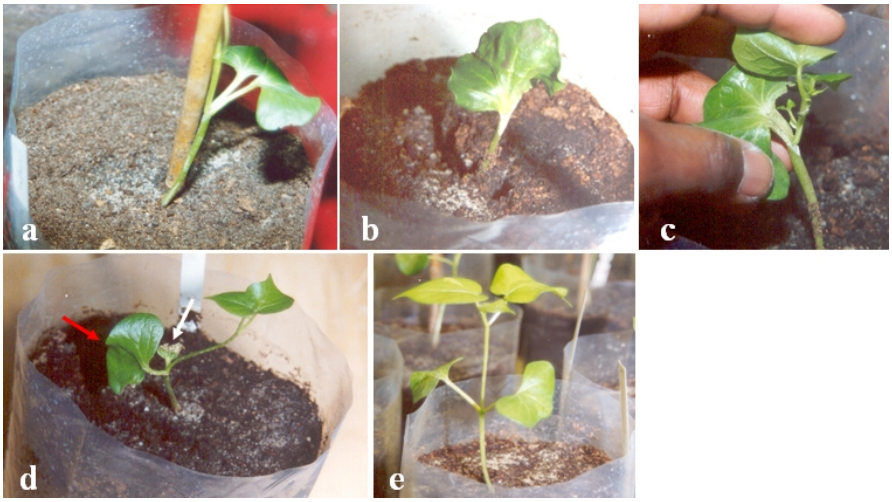

Figure 3. Pictures showing abnormalities observed in some seedlings of the hexaploid at cotyledon leaves stage: (a) a hexaploid seedling showing cotyledonary leaves welded at the level of the petioles; (b), (c) a hexaploid seedling having cotyledonary leaves welded on their entire length (petiole + blade); (d) a hexaploid seedling with a normal cotyledonary leaf (red arrow) and a cotyledonary leaf showing progressive necrosis (white arrow); (e) a hexaploid seedling with normal cotyledonary leaves.

192 cm, while the height of *G. hirsutum* and *G. longicalyx* were on average 82 cm and superior to 300 cm respectively. The hexaploid had 12 to 34 nodes (25 on average) on the main stem. The numbers of nodes on the main stem were on average 11 and 33 for *G. hirsutum* and *G. longicalyx* respectively. The hexaploid plants mostly carried tri-lobated leaves. The size of these leaves varied from 7.4 cm to 14.9 cm with an average of 10.54 cm. *G. hirsutum* presents the largest leaves (23.5 cm on average) with 3 - 5 lobes, while *G. longicalyx* had the smallest leave (6 cm on average) with no lobe (simple leaves). The flowers of the hexaploid plants were a bit smaller than those of *G. hirsutum* but their general aspect was similar. *G. longicalyx* had the smallest flowers. The hexaploid produced relative smaller capsule than *G. hirstum* but bigger than *G. longicalyx*. These results showed that most of the morphological characteristics exhibited by the synthetic allohexaploid hybrid were intermediate between *G. hirsutum* and *G. longicalyx*. This hexaploid hybrid did not generate transgressive morphological characters (characters which show values beyond the range of parental species). [20] studying the synthetic allohexaploid hybrid (*G. hirsutum* x *G. anomalum*)^2 also found that most of morphological characteristics of the hexaploid plants were intermediate between the two parental species. The intermediate morphology of hybrids compared to their parental species is a characteristic observed in several hybrids, since these characters are generally under polygenic control with simple additive effects [34] [35].

3.4. Fertility Analysis

The mean proportion of stainable pollen grains (pollen fertility) of the hexaploid plants was 83% while the pollen fertility of *G. hirsutum* and *G. longicalyx* ap-

proached 100%. The pollen fertility level of the hexaploid is quite good, even if it is relatively lower than that of the parental species. By selfing, the hexaploid gave a mean of 4.39 seeds per pollinated flowers while *G. hirsutum* and *G. longicalyx* produced respectively 34 and 6 seeds per capsule on average (Table 1). These results show the restoration of fertility at the hexaploid level by the doubling of the chromosomes of the sterile triploid hybrid, even if the self-fertility of the parental species was higher. The results of cross-pollinations between the hexaploid and *G. hirsutum* (Table 1) gave practically no seed per capsule, *i.e* 0.018 and 0.3 seed per capsule when the hexaploid was used as female and male respectively. This very low success rate of cross-pollination, despite the good level of pollen fertilities (for both hexaploid hybrid and *G. hirsutum*) is probably due to the presence of incompatibility barriers between the hexaploid hybrid and *G. hirsutum* [36] [37].

3.5. Expression of Fiber Fineness and Resistance to Reniform Nematode Traits

The results of the fiber fineness analysis are presented in Table 2. The hexaploid (*G. hirsutum* × *G. longiclayx*)^2 showed a mean value of ribbon width of 12.53 µm. *G. longicalyx* had the finest fibers with 5.94 µm of ribbon width against 17.765 µm for *G.hirsutum*. These results show the interesting fiber fineness of

Table 1. Selfing of the hexaploid (*G. hirsutum* × *G. longicalyx*)^2 and its parental species, and backcrossing of the hexaploid to *G. hirsutum*.

Type of crossing	No of pollinated flowers	No of seeds harvested	No of seeds per per pollinated flowers
Selfing of *G. hirsutum*	30	1020	34
Selfing of *G. longicalyx*	30	180	6
Selfing of (*G. hirsutum* × *G. longicalyx*)^2	23	101	4.39
(*G. hirsutum* × *G. longicalyx*)^2 ♀ × *G. hirsutum* ♂	57	1	0.018
(*G. hirsutum* × *G. longicalyx*)^2 ♂ × *G. hirsutum* ♀	6	2	0.3

Table 2. Ribbon width of the hexaploid (*G. hirsutum* × *G. longicalyx*)^2 and its parental species.

Genotype	Number of fiber analysed	Ribbon widh (µm) ± standard deviation	Min	Max	LSD grouping
G. hirsutum (cv. C2)	107	17.765 ± 0.207	12.092	24.369	C
G. longicalyx	113	5.940 ± 0.202	4.254	8.862	A
(*G. hirsutum* cv. C2 × *G. longicalyx*)^2	122	12.526 ± 0.180	8.946	16.008	B

G. longicalyx as reported by several authors [4] [12] [13], and its remarkable potential to improve the fiber fineness of *G. hirsutum,* with regard to the expression of this interesting trait in the hexaploid (*G. hirsutum* × *G. longiclayx*)^2.

The data concerning the evaluation of the resistance to the reniform nematode are presented in Table 3. The parental species *G. hirsutum* presented the greatest number of eggs per gram root (205.8 eggs/g root) while the number of eggs per gram root of *G. longicalyx* and the hexaploid were very low, 0 and 3.8 eggs per gram root respectively. Compared to the susceptible *G. hirsutum* species, *G. longicalyx* (0% eggs/g root) and the hexaploid (1.96% eggs/g root) were very resistant. This finding confirms the high resistance to the reniform nematode of the wild African cotton species *G. longicalyx* [14] [15] and shows the inheritance and expression of this interesting trait in the hexaploid.

3.6. Molecular Analysis with AFLP Markers

The results of the AFLP analysis are presented in Table 4. The AFLP electrophoretic profile obtained (Figure 4) revealed a total of 143 loci distributed in 52, 45 and 46 loci for respectively the primer pairs E-ACC/M-CAG, E-ACT/M-CTG and E-ACT/M-CAG. Of the 143 loci revealed, 57 were common to *G. hirsutum* and *G. longicalyx* and therefore monomorphic between these two parental species. All of these 57 monomorphic loci were detected in the hexaploid. In addition to the monomorphic loci, 44 loci were specific to *G. hirsutum* and 42 others were specific to *G. longicalyx*, which makes 86 polymorphic loci in all, representing a percentage of polymorphism of 60.14%. Thus, the AFLP markers

Table 3. Results of the assessment of the resistance to the reniform nematode of the hexaploid (*G. hirsutum* × *G. longicalyx*)^2 and its parental species.

Genotypes	No of Plants	Eggs No per gram root	Percentage of egg per gram root compared to *G. hirsutum* control	Host status
G. hirsutum (cv C2)	5	205.8 ± 7.63	100%	S[a]
G. longicalyx	5	0	0%	HR[b]
Hexaploid	30	3.8 ± 3.11	1.96% ± 1.6	HR

[a]S: susceptible; [b]HR: highly resistant.

Table 4. Results of the analysis of the AFLP electrophoretic profile of the hexaploid (*G. hirsutum* × *G. longicalyx*)^2 and its parental species.

AFLP primer pairs	Total No of bands generated	Total No of *G. hirsutum* bands	No of *G. hirsutum* specific (non specific) bands	No of *G. hirsutum* specific bands present (absent) in the hexaploid	Total No of G. longicalyx bands	No of *G. longicalyx* specific (non specific) bands	No of *G. longicalyx* specific bands present (absent) in the hexaploid	Total No of bands in the hexaploid
E-ACC/M-CAG	52	34	10 (24)	10 (0)	42	18 (24)	16 (2)	50
E-ACT/M-CTG	45	36	21 (15)	21 (0)	24	9 (15)	9 (0)	45
E-ACT/M-CAG	46	31	13 (18)	13 (0)	33	15 (18)	13 (2)	44
Total	**143**	**101**	**44 (57)**	**44 (0)**	**99**	**42 (57)**	**38 (4)**	**139**

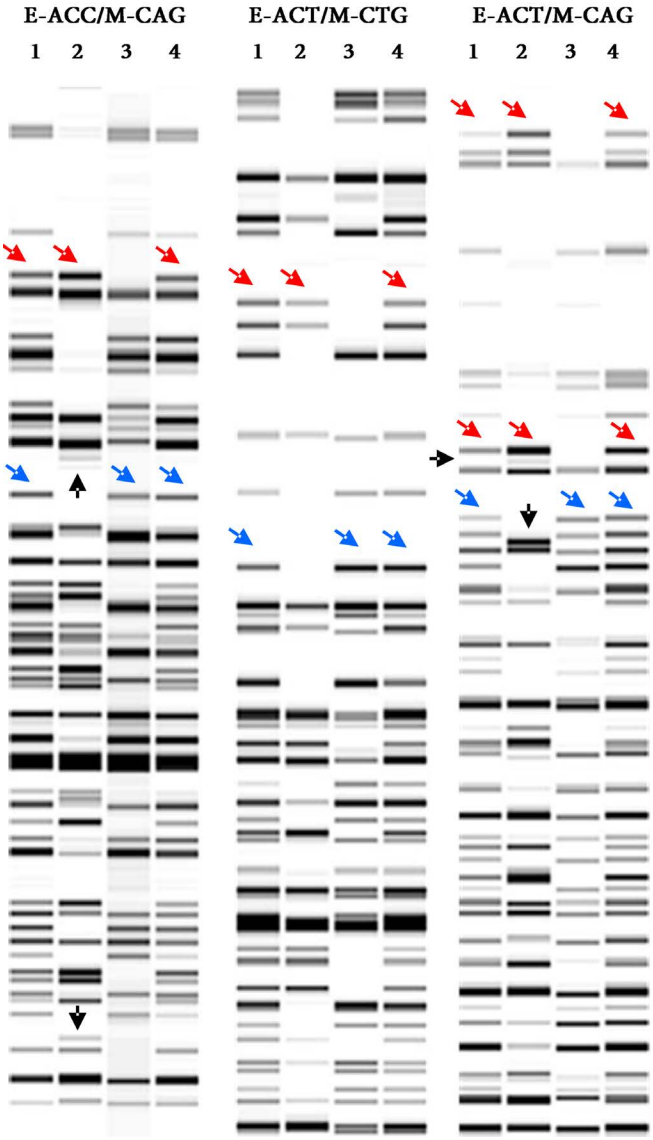

Figure 4. AFLP electrophoretic profile of the hexaploid (*G. hirsutum* × *G. longicalyx*)[2] and its parental species using the three primer pairs E-ACC/M-CAG, E-ACT/M-CTG and E-ACT/M-CAG: 1 = hexaploid#1; 2 = *G. longicalyx*; 3 = *G. hirsutum*; 4 = hexaploid#2. The red arrows show some *G. longicalyx* specific bands; the blue arrows show some *G. hirsutum* specific bands; the black arrows show specific bands *G. longicalyx* that are missing in the hexaploid hybrid.

efficiently discerned differences between the two parental species and distinguished them distinctly from each other. This is consistent with [38] who also highlighted such differences between *G. hirsutum* and *G. longicalyx*, using SNP markers.

All 44 specific loci of *G. hirsutum* and 38 of 42 specific loci of *G. longicalyx* were found in the hexaploid. In total, all the loci revealed in the hexaploid come from *G. hirsutum* and *G. longicalyx*, which confirms the hybrid status of the hexaploid as indicated by cytological analysis. However, 4 of the 42 specific loci

of *G. longicalyx* were missing in the hexaploid (**Figure 4**). [20] studying the synthetic allohexaploid (*G. hirsutum* × *G. anomalum*)^2 also found missing SSR alleles of the wild species *G. anomalum* in the hexaploid. Generally, the main reasons used to explain parental band missings in hybrids are loss of chromosomes or rearrangements of chromosomes. In the present study, the loss of chromosomes cannot explain the missing AFLP loci because the synthetic allohexaploid had the expected number of chromosomes (2n = 78). The explanation of chromosomal rearrangements is also unlikely since the two different hexaploid plants used for the molecular analysis had exactly the same missing loci. It is unlikely that there will be exactly the same recombination in two different plants. A slight genetic differentiation between the *G. longicalyx* plant used to develop the hexaploid hybrid and the *G. longicalyx* plant used in this molecular analysis could likely be the reason for the missing loci in the hexaploid hybrid. Indeed, intraspecific differentiation exists in wild cotton species. For example, [39] using AFLP data, reported intraspecific variation (in terms of percentage of polymorphic fragments) in four species of cotton (*G. aridum*, *G. laxum*, *G. lobatum*, and *G. schwendimanii*). Anyway, phenotypic analysis showed that the current synthetic allohexaploid hybrid had the characteristics of interest (resistance to reniform nematode and fineness of the fibers) sought in *G. longicalyx*. Therefore, this hexaploid material is perfectly suited for use in a cotton improvement program.

4. Conclusion

The present study demonstrated the hybridity and hexaploid status of the genotype studied using cytological and molecular marker methods. Moreover, this synthetic allohexaploid hybrid exhibited the useful traits of the African wild diploid species *G. longicalyx* with regard to the fineness of the fibers and the resistance to reniform nematode. This synthetic allohexaploid hybrid represents very interesting genetic stocks that can be used as a bridge for the transfer of useful agronomic traits from wild species to upland cotton varieties.

Conflicts of Interest

The authors declare that there is no conflict of interest.

References

[1] Ayubov, M.S., Abdurakhmonov, I.Y., Sripathi, V.R., Saha, S., Norov, T.M., Buriev, Z.T., Shermatov, S.E., Ubaydullaeva, K.A., McCarty, J.C., Deng, D.D. and Jenkins, J.N. (2018) Recent Developments in Fiber Genomics of Tetraploid Cotton Species. Intechopen, London. https://doi.org/10.5772/intechopen.72922

[2] Fang, L., Gong, H., Hu, Y., Liu, C., Zhou, B., Huang, T., *et al.* (2017) Genomic Insights into Divergence and Dual Domestication of Cultivated Allotetraploid Cottons. *Genome Biology*, **18**, 33. https://doi.org/10.1186/s13059-017-1167-5

[3] Wu, Y., Liu, F., Yang, D.G., Li, W., Zhou, X.J., Xiao, Y.P., *et al.* (2018) Comparative Chloroplast Genomics of *Gossypium* Species: Insights into Repeat Sequence Varia-

tions and Phylogeny. *Frontiers in Plant Science*, **9**, 376. https://doi.org/10.3389/fpls.2018.00376

[4] Konan, N.O. and Mergeai, G. (2020) Relationship between Meiotic Behaviour and Fertility in Backcross-1 Derivatives of the [(*Gossypium hirsutum* × *G. thurberi*)2 × *G. longicalyx*] Trispecies Hybrid. *Comparative Cytogenetics*, **14**, 75-95. https://doi.org/10.3897/CompCytogen.v14i1.47231

[5] Wendel, J.F. and Grover, C.E. (2015) Taxonomy and Evolution of the Cotton Genus, *Gossypium*. In: Fang, D.D. and Percy, R.G., Eds., *Cotton*, American Society of Agronomy Inc., Madison, 25-44. https://doi.org/10.2134/agronmonogr57.2013.0020

[6] Gallagher, J.P., Grover, C.E., Rex, K., Moran, M. and Wendel, J.F. (2017) A New Species of Cotton from Wake Atoll, *Gossypium stephensii* (Malvaceae). *Systematic Botany*, **42**, 115-123. https://doi.org/10.1600/036364417X694593

[7] Wang, K., Wendel, J.F. and Hua, J. (2018) Designations for Individual Genomes and Chromosomes in *Gossypium*. *Journal of Cotton Research*, **1**, 3. https://doi.org/10.1186/s42397-018-0002-1

[8] Ijaz, B., Zhao, N., Kong, J. and Hua, J. (2019) Fiber Quality Improvement in Upland Cotton (*Gossypium hirsutum* L.): Quantitative Trait Loci Mapping and Marker Assisted Selection Application. *Frontiers in Plant Science*, **10**, 1585. https://doi.org/10.3389/fpls.2019.01585

[9] Shim, J., Mangat, P.K. and Angeles-Shim, R.B. (2018) Natural Variation in Wild *Gossypium* Species as a Tool to Broaden the Genetic Base of Cultivated Cotton. *Journal of Plant Science*: *Current Research*, **2**, 005.

[10] Islam, M.S., Zeng, L., Thyssen, G.N., Delhom, C.D., Kim, H.J., Li, P. and Fang, D.D. (2016) Mapping by Sequencing in Cotton (*Gossypium hirsutum*) Line MD52ne Identified Candidate Genes for Fiber Strength and Its Related Quality Attributes. *Theoretical and Applied Genetics*, **129**, 1-6. https://doi.org/10.1007/s00122-016-2684-4

[11] Mergeai, G. (2006) Interspecific Introgressions in Cotton. *Cahiers Agricultures*, **15**, 135-143.

[12] Gotmare, V., Singh, P. and Tule, B.N. (2000) Wild and Cultivated Species of Cotton. CICR Technical Bulletin No. 5, CICR, Mumbai, 1-21.

[13] Nacoulima, L.N., Diou, F.H., Konan, N.O. and Mergeai, G. (2016) Production of New Cotton Interspecific Hybrids with Enhanced Fiber Fineness. *Journal of Agricultural Science*, **8**, 46-56. https://doi.org/10.5539/jas.v8n2p46

[14] Yik, C.P. and Birchfield, W. (1984) Resistant Germplasm in *Gossypium* Species and Related Plants to *Rotylenchulus reniformis*. *Journal of Nematology*, **16**, 146-153.

[15] Konan, N.O., De Proft, M., Ruano, O., Baudoin, J.-P. and Mergeai, G. (2014) A Screening Procedure for Evaluating Cotton for *Rotylenchulus reniformis* Resistance in Controlled Conditions. *Tropicultura*, **32**, 3-9.

[16] Konan, N.O. and Mergeai, G. (2007) Breeding Possibilities of the Main Cultivated Cotton Species (*Gossypium hirsutum* L.) for the Resistance to Reniform Nematode (*Rotylenchulus reniformis* Linford et Oliveira). *Biotechnology*, *Agronomy*, *Society and Environment*, **11**, 159-171.

[17] Sarr, D. and Mergeai, G. (2009) Genetic Broadening of the Main Cultivated Cotton Species *Gossypium hirsutum* L. by Creation and Exploitation of Monosomic Alien Addition Lines. *Biotechnology*, *Agronomy*, *Society and Environment*, **13**, 187-201.

[18] Konan, N.O., D'Hont, A., Baudoin, J.P. and Mergeai, G. (2007) Cytogenetics of a New Trispecies Hybrid in Cotton: [(*Gossypium hirsutum* L. x *G. thurberi* Tod.)^2 x

G. longicalyx Hutch. & Lee]. *Plant Breeding*, **126**, 176-181. https://doi.org/10.1111/j.1439-0523.2007.01325.x

[19] Mergeai, G. (2004) Forty Years of Genetic Improvement of Cotton through Interspecific Hybridisation at Gembloux Agricultural University: Achievement and Prospects. *Proceedings of the World Cotton Research Conference*, Cape Town, 9-13 March 2003, 120-133.

[20] Zhang, X., Zhai, C., He, L., Guo, Q., Zhang, X., Xu, P., *et al.* (2014) Morphological, Cytological and Molecular Analyses of a Synthetic Hexaploid Derived from an Interspecific Hybrid between *Gossypium hirsutum* and *Gossypium anomalum*. *The Crop Journal*, **2**, 272-277. https://doi.org/10.1016/j.cj.2014.06.009

[21] Roehrich, O. (1947) General Method for the Study of Technological Characteristics of Plant Textile Fibers. *Coton et Fibres Tropicales*, **2**, 81-89.

[22] Vroh Bi, I., Baudoin, J.-P., Hau, B. and Mergeai, G. (1999) Development of High-Gossypol Cotton Plants with Low Gossypol Seeds Using Trispecies Bridge Crosses and *in Vitro* Culture of Seed Embryos. *Euphytica*, **106**, 243-251. https://doi.org/10.1023/A:1003539924238

[23] Dhooghe, E., Van-Laere, K., Eeckhaut, T., Leus, L. and Van-Huylenbroeck, J. (2011) Mitotic Chromosome Doubling of Plant Tissues *in Vitro*. *Plant Cell Tissue and Organ Culture*, **104**, 359-373. https://doi.org/10.1007/s11240-010-9786-5

[24] Cohen, H., Fait, A. and Tel-Zur, N. (2013) Morphological, Cytological and Metabolic Consequences of Autopolyploidization in *Hylocereus* (Cactaceae) Species. *BMC Plant Biology*, **13**, Article No. 173. https://www.biomedcentral.com/1471-2229/13/173 https://doi.org/10.1186/1471-2229-13-173

[25] Castro, M., Castro, S. and Loureiro, J. (2018) Production of Synthetic Tetraploids as a Tool for Polyploid Research. *Web Ecology*, **18**, 129-141. https://doi.org/10.5194/we-18-129-2018

[26] Beest, M., Le Roux, J.J., Richardson, D.M., Brysting, A.K., Suda, J., Kubesová, M. and Pysek, P. (2012) The More the Better? The Role of Polyploidy in Facilitating Plant Invasions. *Annals of Botany*, **109**, 19-45. https://doi.org/10.1093/aob/mcr277

[27] Hosseini, H., Chehrazi, M., Sorestani, M. and Ahmadi, D. (2013) Polyploidy and Comparison of Diploid and Autotetraploid Seedling of Madagascar Periwinkle (*Catharanthus roseus* cv. alba). *International Research Journal of Applied and Basic Sciences*, **4**, 402-406.

[28] Chae, W.B., Hong, S.J., Gifford, J.M., Lane Rayburn, A., Widholm, J.M. and Juvik, J.A. (2013) Synthetic Polyploid Production of *Miscanthus sacchariflorus*, *Miscanthus sinensis*, and *Miscanthus* x *giganteus*. *GCB Bioenergy*, **5**, 338-350. https://doi.org/10.1111/j.1757-1707.2012.01206.x

[29] Münzbergová, Z. (2017) Colchicine Application Significantly Affects Plant Performance in the Second Generation of Synthetic Polyploids and Its Effects Vary between Populations. *Annals of Botany*, **120**, 329-339. https://doi.org/10.1093/aob/mcx070

[30] Liu, B. and Wendel, J.F. (2003) Epigenetic Phenomena and the Evolution of Plant Allopolyploids. *Molecular Phylogenetics and Evolution*, **29**, 365-379. https://doi.org/10.1016/S1055-7903(03)00213-6

[31] Doyle, J.J., Flagel, L.E., Paterson, A.H., Rapp, R.A., Soltis, D.E., Soltis, P.S. and Wendel, J.F. (2008) Evolutionary Genetics of Genome Merger and Doubling in Plants. *Annual Review of Genetics*, **42**, 443-461. https://doi.org/10.1146/annurev.genet.42.110807.091524

[32] Padmanaban, S., Zhang, P., Hare, R.A., Sutherland, M.W. and Martin, A. (2017) Pentaploid Wheat Hybrids: Applications, Characterisation, and Challenges. *Frontiers in Plant Science*, **8**, 358. https://doi.org/10.3389/fpls.2017.00358

[33] Chu, C.-G., Faris, J., Friesen, T. and Xu, S. (2006) Molecular Mapping of Hybrid Necrosis Genes *Ne*1 and *Ne*2 in Hexaploid Wheat Using Microsatellite Markers. *Theoretical and Applied Genetics*, **112**, 1374-1381. https://doi.org/10.1007/s00122-006-0239-9

[34] Rieseberg, L.H., Kim, S., Randell, R.A., Whitney, K.D., Gross, B.L., Lexer, C. and Clay, K. (2007) Hybridization and the Colonization of Novel Habitats by Annual Sunflowers. *Genetica*, **129**, 149-165. https://doi.org/10.1007/s10709-006-9011-y

[35] López-Caamal, A. and Tovar-Sánchez, E. (2014) Genetic, Morphological, and Chemical Patterns of Plant Hybridization. *Revista Chilena de Historia Natural*, **87**, 16. https://www.revchilhistnat.com/content/87/1/16 https://doi.org/10.1186/s40693-014-0016-0

[36] Mehetre, S.S., Gawande, V.L. and Aher, A.R. (2002) Imcompatibility in Wide Hybridization of *Gossypium* Species: Causes and Remedies—A Review. *Journal of Cotton Research and Development*, **16**, 111-124.

[37] Tonosaki, K., Osabe, K., Kawanabe, T. and Fujimoto, R. (2016) The Importance of Reproductive Barriers and The Effect of Allopolyploidization on Crop Breeding. *Breeding Science*, **66**, 333-349. https://doi.org/10.1270/jsbbs.15114

[38] Hinze, L.L., Hulse-Kemp, A.M., Wilson, I.W., *et al*. (2017) Diversity Analysis of Cotton (*Gossypium hirsutum* L.) Germplasm Using the CottonSNP63K Array. *BMC Plant Biology*, **17**, Article No. 37. https://doi.org/10.1186/s12870-017-0981-y

[39] Álvarez, I. and Wendel, J.F. (2006) Cryptic Interspecific Introgression and Genetic Differentiation within *Gossypium aridum* (Malvaceae) and Its Relatives. *Evolution*, **60**, 505-517. https://www.jstor.org/stable/4095313 https://doi.org/10.1111/j.0014-3820.2006.tb01132.x