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Introgression of the *low-gossypol seed & high-gossypol plant* trait in upland cotton: Analysis of [(*Gossypium hirsutum* × *G. raimondii*)^2 × *G. sturtianum*] trispecific hybrid and selected derivatives using mapped SSRs

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Abstract In order to select genotypes of *Gossypium hirsutum* genetically balanced and expressing the *low-gossypol seed & high-gossypol plant* trait introgressed from the Australian wild diploid species *G. sturtianum*, the [(*G. hirsutum* × *G. raimondii*)^2 × *G. sturtianum*] triple hybrid was backcrossed to *G. hirsutum* and autopollinated to produce backcross and selfed progenies. Two hundred and six mapped SSR markers of *G. hirsutum* were used to monitor the introgression of SSR alleles specific to *G. sturtianum* and *G. raimondii* in the selected progenies. A high level of heterozygosity, varying from 25 to 100%, was observed for all *G. sturtianum*-specific SSR markers conserved in the most advanced progenies. These results indicate the existence of segregation distortion factors that are associated with the genes controlling the researched trait. This study represents a starting point to map the genes involved in the expression of the trait and better understand its genetic determinism.

Keywords *Gossypium* · Introgression · Microsatellites · Gossypol · *Glandless seed* · Interspecific hybrid

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Introduction

The cotton genus *Gossypium* contains 49 diploid and tetraploid species distributed worldwide in both tropical and subtropical areas (Fryxell 1992). The 44 diploid species ($2n = 2x = 26$) are grouped into eight different cytotypes designated A–G and K (Endrizzi et al. 1985; Stewart 1995). They count two cultivated species, *G. herbaceum* and *G. arboreum*. The five tetraploid species (designated (AD)) contain two distinct subgenomes which are related to the A genome of the Asiatic cultivated diploid species and D genome of the American wild diploid species (Wendel and Cronn 2003; Endrizzi et al. 1985). They include two cultivated species, *G. barbadense* and *G. hirsutum*, the latter (upland cotton) being the most important.

Cotton is the world's leading natural fiber crop but also ranks high among the food crops (Lusas and Jividin 1987). For every kilogram of fiber, the plant

0.1 μg/mg seed gossypol, while maintaining gossypol and related terpenoids in the foliage and floral parts of the plant. This technique represents another possible way to modify seed gossypol, but further testing is still needed to confirm the expression in advanced generations of the *glandless-seed and glandled-plant* trait.

The present study was initiated in order to monitor the introgression of chromosome segments from the wild species *G. sturtianum* and *G. raimondii* in selected advanced generations of the [(*G. hirsutum* × *G. raimondii*)^2 × *G. sturtianum*] (HRS) trispecific hybrid with the aim to map the *low-gossypol seed & high-gossypol plant* trait.

Materials and methods

Plant material

All the plants used for the creation of the trispecific allotetraploid hybrid HRS [(*G. hirsutum* × *G. raimondii*)^2 × *G. sturtianum*], [$A_hD_hD_5C_1$] are maintained in the cotton collection of the Gembloux Agricultural University (GAU). Two cultivars of *G. hirsutum* L. $2(A_hD_h)_1$ (NC8 and C2), selected in the Democratic Republic of Congo, one accession of *G. raimondii* Ulbr. ($2D_5$) and one accession of *G. sturtianum* Willis. ($2C_1$) were used for the creation of the HRS hybrid according to the pseudophyletic introgression method (Mergeai 2006). This method ends with the creation of trispecific hybrids involving *G. hirsutum* and two diploid species. Tetraploid *Gossypium hirsutum* is crossed directly with one of the diploid parents, creating a triploid hybrid. Chromosome doubling gives a fertile allohexaploid which is crossed to the other diploid parent, resulting in the allotetraploid trispecific HRS hybrid.

Variety 'STAM F' from Togo was used for backcrossing the HRS hybrid. The scheme to create the trispecific hybrid is detailed in Vroh Bi et al. (1998). Plants selected in the first and next backcross generations were, euploids ($2n = 4x = 52$) and showed a high frequency of chromosome pairing and chiasmata. Figure 1 presents the crossing scheme and generations studied.

One BC_2S_1 plant and one BC_3 plant hybrid produced seeds with very different levels of gossypol glands and were chosen for their ability to give segregating progenies for this trait (Mergeai et al. 1997). These two plants were both self pollinated and backcrossed to *G. hirsutum* cultivar STAM F to produce subsequent progenies. A distilled water solution of growth regulators (100 mg l^{-1} naphtoxyacetic acid + 50 mg l^{-1} gibberellic acid) was applied on the ovary just after pollination to limit the shedding of bolls. Only plants resulting from seeds having the lowest level of gossypol glands visible on their kernel wall and producing the highest proportion of seeds presenting the "low gossypol seed and high gossypol plant" trait were retained in each generation. All the plants studied in this work were cultivated under greenhouses condition at GAU.

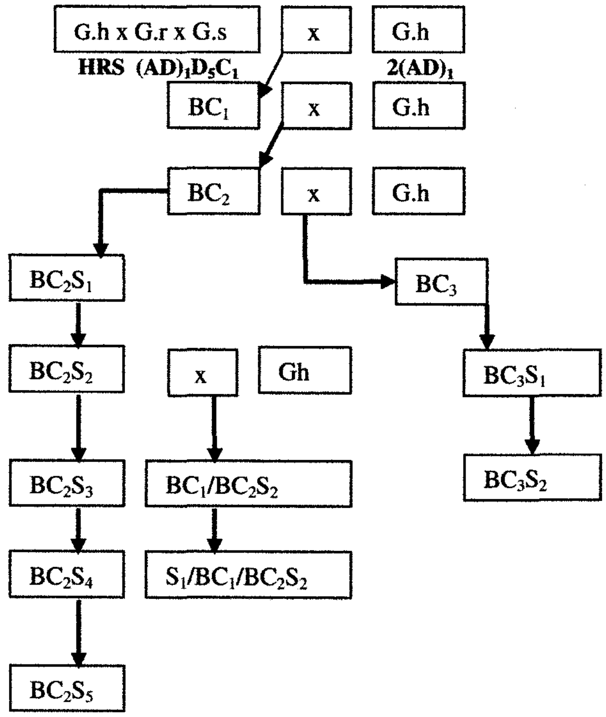

Fig. 1 Selection scheme of HRS derivatives expressing the low-gossypol seed and high-gossypol plant trait. *G. h G. hirsutum*, *G. r G. raimondii*, *G. s G. sturtianum*, *HRS* allotetraploid trispecies hybrid

Assessment of gossypol content and external gossypol gland density of the seeds

The external gland density (EGD) was assessed after removing seed integument on soaked kernels according to a visual scale ranging from 0 for totally glandless to 10 for highly *glanded* seeds. *Glandless* or nearly *glandless* BC_2S_2 seeds evaluated this way were cultured in vitro on the medium of Stewart and Hsu (1977) in a growth chamber regulated at 27°C, with 12 h photoperiod (10 μ Einstein m^{-2} s^{-1}). Seeds belonging to the subsequent generations were sown directly in a substrate made of sand, peat and compost in equal proportions.

The gossypol content of the seeds produced by the low EGD genotypes was assessed seed by seed using the destructive method developed by Benbouza et al. (2002). This method of indirect quantification of the seed gossypol content (SGC) is based on the relation between gossypol content (in % of seed kernel mass) and the number of glands per seed section, following model: $\%G = b \times (N/S)$; where $\%G$ is the content of gossypol in %, N is the number of gossypol glands per seed section, S is the area of the seed section expressed in mm^2, and b is the regression coefficient calculated for the progeny of a particular genotype. Seeds are cut in two longitudinal sections after removal of the teguments in order to count the number of glands (Fig. 2). These operations were carried out with a Nikon Eclipse E800 light and fluorescent microscope (Nikon, Tokyo, Japan) using a JVC-3-CCD colour video camera (JVC, Tokyo, Japan) and the Archive Plus program of Sony (Sony Electronics, NJ, Park Ridge, USA) to capture and analyse the images. On the basis of the results obtained by Benbouza et al. (2002), the values of b used in our study for the assessment of $\%G$ were 0.1831 for *G. hirsutum* STAM F control, 0.1217 for the progeny of the BC_2S_1/09 plant and 0.1701 for the progeny of the BC_3/09 plant.

DNA isolation and quantification

DNA was extracted from one to two grams of fresh young leaf using the protocol developed by Benbouza et al. (2006a). DNA was also extracted from BC_2S_5 seeds to increase the number of analysed individuals according to the method outlined by Wang et al.

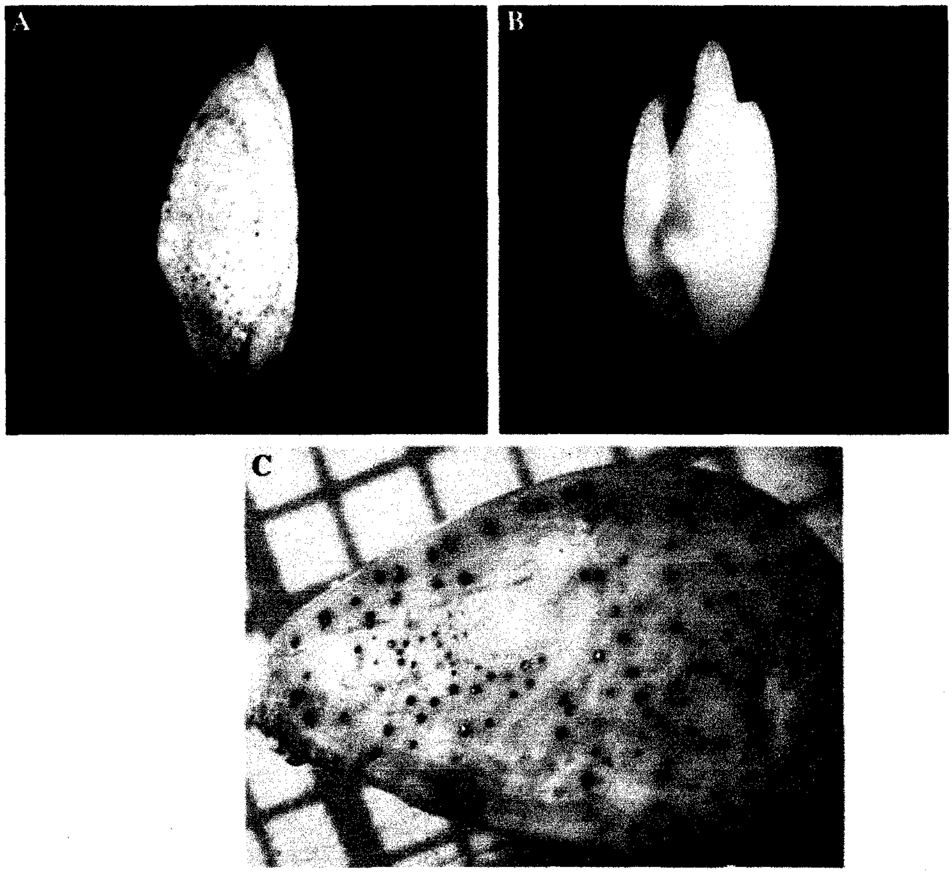

Fig. 2 **a, b** Evaluation of the external gossypol gland density (*EGD*) for *G. hirsutum* and *G. sturtianum*, respectively. **c** Evaluation of the seed gossypol content (*SGC*)

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(1993). In CIRAD DNA concentration was quantified using a Fluoroskan Ascent FL (Thermo Fisher Scientific Inc., Waltham, USA).

Microsatellite marker analysis

Simple sequence repeat (SSR) markers used were developed at Brookhaven National Laboratory (prefix BNL) and at CIRAD (prefix CIR). The SSRs was chosen from preliminary screenings for their ability to reveal polymorphic alleles specific to either diploid parental species.

Molecular analyses were partly conducted at CIRAD (generations BC_1, BC_2, BC_2S_1, $S_1BC_1BC_2S_2$, BC_3, BC_3S_1, and BC_3S_2) and GAU (generations BC_2S_5 and $S_2BC_1BC_2S_2$) as described in Risterucci et al. (2000) and Liu et al. (2000) respectively. Radioactive labelling was used in CIRAD while a silver staining revelation technique was used at GAU (Benbouza et al. 2006b).

Each of the thirteen homoeologous chromosome pairs of the cotton genome map was screened with a minimum of four SSRs except for the c16 which was screened with three SSRs.

All microsatellites used covered almost the entire length of the chromosomes except for c4 and c16 in which only 108.9 cM out of 189.5 cM and 62.8 cM out of 165.8 cM were covered respectively. On an average, there were eight markers per chromosome, varying from 3 on c16 to 18 on c5.

Totally, 206 SSRs were tested on 25 DNA samples including the HRS hybrid, *G. sturtianum*, *G. raimondii*, *G. hirsutum* cultivars C2, NC8, STAM F, and TM1 standard and the following selected progenies: BC_1 (1 genotype) BC_2 (1 genotype), BC_2S_1 (1 genotype), BC_2S_5 (5 genotypes), $BC_1BC_2S_2$ (2 genotypes), $S_1BC_1BC_2S_2$ (2 genotypes), $S_2BC_1BC_2S_2$ (2 genotypes), BC_3 (1 genotype), BC_3S_1 (1 genotype), and BC_3S_2 (2 genotypes).

Results

Production of introgressed materials

The plants of interest were selected according to a two step approach. A non destructive assay (gland density score on kernel surface) was carried out first to identify among the seeds produced by the plants selected in the previous generation the ones presenting a reduced density of gossypol glands on their kernel. The plants issued from these seeds were later screened for their ability to express the *low-gossypol seed* and *high-gossypol plant* trait. For this purpose, a part of the seeds they produced by selfing was sacrificed to quantify their gossypol content using the destructive method developed by Benbouza et al. (2002) and the density of pigment glands on their aerial parts was visually assessed. Only glanded plants able to produce regularly totally or almost totally glandless seed were finally selected. Special efforts were necessary to produce viable progenies in the early derivative generations. No seed were produced by selfing the HRS hybrid and its direct backcross progeny. On average, despite the application of growth regulator to prevent boll shedding after pollination, about 15 crosses were necessary to obtain one seed with both HRS and BC_1 genotypes. Initially, all the BC_1 seeds produced by the HRS trispecific hybrid were planted in Jiffy pots. This practice resulted in a very low survival rate of the planted seeds. Less than 25% of these first BC_1 seeds gave rise to adult plants. The rest, including all the first totally glandless seeds produced by HRS hybrid, did not germinate or died at a very early stage. To improve their survival rate, all the BC_1 seeds were then cultivated in vitro on the rooting medium developed by Stewart and Hsu (1977). This allowed the rescue of about two-third of the genotypes. In the subsequent generations, in vitro cultivation of the low gossypol seed mature embryos was still necessary until the BC_2S_2. The fertility of the backcross derivatives improved markedly with advancing generations. Pollen stainability was very low in the trispecific hybrid and its BC_1 derivatives (less than 10%) but increased to about 60% in fertile BC_2 plants and was between 95 and 100% in all the subsequent generations. On an average, four crosses were necessary to obtain one BC_2 seed while one backcross of a fertile BC_2 plant gave about 5 BC_3 seeds. BC_2S_1 plants were less fertile than BC_3 materials (about two crosses were necessary to get one seed). The crossing success rate increased to about a dozen seeds per cross for the most advanced generation (BC_2S_5 and $S_2BC_1BC_2S_2$) All the selected genotypes were multiplied by grafting in at least two copies in order to increase the number of seeds they produced before assessing the inhibition of the seed gossypol synthesis using the SGC method.

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derived from totally glandless seeds in the backcrossed progeny of the *G. hirsutum* × *G. sturtianum* pentaploid carried several supernumerary chromosomes of the donor species. These data also prove the soundness of the triple hybrid strategy that was used to introgress the researched trait into *G. hirsutum*. In such hybrids the chromosomes of the American dipolid bridge species (*G. raimondii*) should pair with D_h subgenome while the chromosomes of the Australian diploid donor species (*G. sturtianum*) should pair preferentially with the A_h subgenome of *G. hirsutum*, allowing the simultaneous recombination segments of the chromosomal segments from the Australian donor species involved in the control of the researched trait.

The shape of the seed gossypol content frequency distributions observed for the successive generations of selected HRS derivatives were generally in agreement with the hypothesis that more than one gene is involved in the control of the repression of pigment gland morphogenesis in the seed. The genotypes containing all *G. sturtianum* DNA fragments involved in the determinism of the researched trait should be the ones that are able to express it at the highest level. After more than five generations of backcross and selfing, a high level of segregation for gossypol content was still observed in the seeds produced by the selected HRS derivatives. This is probably due to the heterozygous state of the *G. sturtianum* genes controlling the researched trait. The high frequencies of heterozygosity we observed for most of the conserved *G. sturtianum*-specific SSR alleles confirm this hypothesis.

The variation in gossypol content frequency distributions according to the generation of the HRS derivatives can also be due to several factors that might interact with the expression of the genes of *G. sturtianum* that repress the synthesis of gossypol only in the seed.

An important influence of environmental factors on the seed gossypol content was observed by Pons et al. (1953) who compared gossypol content of seeds produced by eight upland cotton varieties in 13 different environments during three consecutive years. According to the location and the year of production, these authors observed for the same *G. hirsutum* variety (Acala 4–42), a variation of the kernel gossypol content ranging from 0.39 to 1.17%. They also found that gossypol content in the kernels was significantly correlated with the temperature and the rainfall, and that individual cotton varieties differed in their response to environmental factors. As the seeds we used to quantify the gossypol content and establish the frequency distributions presented in Table 1 were not all produced the same year, it is possible that their gossypol content was influenced by the important variations in environment conditions that occurred in Gembloux during their period of production. These changes mainly concerned the temperature and the cumulated amount of solar radiation received by the plants during summer.

The high level of residual heterozygosity can due to a number of factors. The genetic background in which the chromosome fragments of *G. sturtianum* were introgressed can influence the expression of the *low-gossypol seed and high gossypol plant* trait. This genetic background acts by repressing or modifying genes. The transfer of alleles between species can lead to a break up of the original system (alleles of modifying genes) and result in a reduction of the efficiency of the alleles in the new genetic background (Pauly 1979).

The gene order and spacing on *G. sturtianum* chromosomes may not be conserved and therefore decrease the opportunity for recombination. In this study even in the most advanced selected genotypes, 10 *G. sturtianum* specific SSR fragments were detected on 3 homoeologous chromosomes pairs (c2, c3 and c6–c25). In addition even after several generations of selfing, there was no recombination observed between BNL3436 and BNL1153 on chromosome c25 although these two loci are reported to be separated by 64 cM on the *G. hirsutum* map (Lacape et al. 2003).

In crop species, both inversion and translocation events have been implicated in the genome rearrangements (Livingstone et al. 1999). Brubaker et al. (1998), while developing a comparative RFLP map of the allotetraploid cotton and its diploid progenitors detected 19 loci order differences. The observed inversions were not fully conserved and two reciprocal translocations were confirmed between allotetraploid A_h genome chromosomes, as was a translocation between the two existent A genome diploids. Similar observations were outlined by Rong et al. (2004) when mapping diploid (D) and tetraploid genome (A_hD_h). They confirmed two reciprocal translocations and several inversions between A_h chromosomes.

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The high frequencies of heterozygosity of *G. sturtianum* SSR loci conserved after five generations of selfing in the BC_2S_5 progenies, indicate that the cytogenetic/genetic conditions for obtaining homozygosity at high frequencies were not met. Segregation distortion (non-Medelian inheritance) and restricted recombination are often found in the mosaic genomes of interspecific hybrid populations (Jiang et al. 2000). Both structural and genic mutations accumulated by species prior to hybridization appear to play a role in non-Medelian inheritance (Rieseberg et al. 1995). Mutations that have accumulated in divergent lineages may be beneficial or benign in their native background, but harmful in alien genetic context. The way these genetic changes interact negatively with the genetic background of the recipient species varies according to their nature. Some act directly during the gamete formation, other induce direct hybrid lethality when present in a heterozygous state, and some need to be present in a homozygous state to cause partial (sublethal and subvital genes) or full (lethal genes) destruction of the zygotes or the seedlings (Lynch and Force 2000). Although F_1 sterility or inviability is a common feature of wide interspecific crosses, small introgressions often have indiscernible heterozygous effects while being lethal or sterilizing in the homozygous state (Turelli and Orr 2000).

Birhman and Hosaka (2000) outlined self-incompatibility and zygote selection, which cause unequal segregation of alleles. Preferential transmission through male or female gametes, or both, has been noted for monosomic alien addition chromosomes introgressed into a cultivated crop species background (Maan 1975). In most instances, the preferential transmission is caused by a single gene located on the alien chromosome (Maguire 1963). When segregation distorters or *Gc* genes occur, one of the alleles at heterozygous loci transmits to the progeny at higher frequencies than the expected Mendelian ratio (Sandler et al. 1959). During meiosis, alien *Gc* genes, in the hetero- or hemizygous state, induce breakage in chromosomes not carrying the genes. The gametes with the broken chromosomes are deficient for some loci and are often unviable. The viable gametes will be those carrying the gametocidal alien chromosome (Endo 1979; Nasuda et al. 1998). Rick (1966) has reported gametes eliminator allele (*Ge*) in tomato, which causes abortion of gametes because of allelic interaction. *Ge* allele induces abortion of the gametes carrying the opposite allele, although the homozygote shows no adverse effect on the formation of the gametes. Our results indicate the presence of the alien SSR markers, BNL3436 and BNL1153, mapped on the c6–c25 linkage groups, in all HRS progenies, from the BC_1 to $S_1/BC_1/BC_2S_2$, sampled in our study. Therefore, it is possible that such gametocide genes may exist on at least one of the *G. sturtianum* chromosome fragments introgressed in HRS progeny. Becerra and Brubaker (2007) proposed the possible presence of a gametocidal chromosome in *G. australe* species when analysing the frequency of alien chromosome transmission in a *Gossypium* hexaploid bridging population. The same gametocidal genes may exist in *G. sturtianum* species. In cotton, preferential transmission of an additional Austrálian diploid species chromosome was mentioned by Rooney and Stelly (1991) and Ahoton et al. (2004). Vroh Bi et al. (1999b) observed that out of 70 species-specific AFLP loci of the donor parent *G. sturtianum*, four were systematically present in all the backcross progenies of two tri-species hybrids [(*G. hirsutum* × *G. raimondii*)$_2$ × *G. sturtianum*] (HRS) and [(*G. raimondii* × *G. sturtianum*)^2 × *G. hirsutum*] (TSH) suggesting that these fragments were located on chromosomes that were preferentially transmitted.

One of the most important barriers that prevent the development of an interspecific hybrid-derived population in cotton is the hybrid lethality. Several species of the A and D genomes (*G. davidsonii* Kell., *G. klotzchianum* Anders., *G. gossypioïdes* (Ulbr.) Stendl. and *G. arboreum* L. race *sanguineum*) present two complementary genes of lethality which condition the death of hybrid embryos or seedlings produced with tetraploid cotton plants (Lee 1982; Rooney and Stelly 1989; Percival et al. 1999). Normally, this type of gene should not be present on the *G. sturtianum* conserved chromosome fragments of the selected HRS hybrid derivatives because their simple presence should have prevented the development of any hybrid between the donor and the recipient species.

Functional lethality due to the presence in the homozygote state of recessive alien lethal alleles was observed in an interspecific hybrid of tomato by Bernacchi and Tanksley (1997). Such genes might be present on the chromosome fragments of

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G. sturtianum introgressed in the most advanced generations of the selected derivatives of HRS.

The mapped SSRs used here were initially chosen on the "A-genome" of modern tetraploid cotton based on (1) the higher pairing affinity of the donor C chromosomes (large size) for A chromosomes (medium size) than for D chromosomes (small size) (Endrizzi et al. 1985); and (2) the greater efficiency of the seed gossypol gland repression mechanism in the wild Australian species against the "A" genome carrying the Gl_2 allele determining seed gossypol gland density than the "D" genome (Mergeai 1992). Considering these two factors, it was expected that the A_h chromosomes of *G. hirsutum* would interact and more likely pair with C_1 chromosomes of *G. sturtianum*. The higher level of introgression observed for *G. raimondii* chromosome fragments compared to *G. sturtianum* where selection pressure was applied to retain only the individuals expressing the researched trait supports the soundness of this hypothesis.

For our future investigations, genomic in situ hybridization (GISH) analyses on the selected materials will be used to measure the amount of introgression and to localize the conserved alien fragments. Cytological analyses will permit us to observe and to score chiasmata associations between cytologically marked chromosomes. Further investigation on populations obtained by crossing the introgressed stocks as male and female parents with *G. hirsutum* varieties will be realized to better understand the segregation distortions observed in our study. The results of these investigations should help identifying the best solutions to break the inhibitory linkages that seem to exist between these segregation distortion factors and the genes controlling the inhibition of the gossypol synthesis only in the seed.

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