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# Introgression of the Glanded-Plant and Glandless-Seed Trait from *Gossypium sturtianum* Willis into Cultivated Upland Cotton Using Ovule Culture[1]

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## ABSTRACT

**Introgression of the glanded-plant/glandless-seed trait from *Gossypium sturtianum* Willis [$2n = 2x = 26$; $C_1$ genome] into upland cotton, *G. hirsutum* L. [$2n = 4x = 52$; $(AD)_1$ genomes] has not been successful using conventional hybridization techniques beyond the first backcross ($BC_1$) pentaploid generation [$2n = 5x = 65$; genomic formula $2(AD)_1C_1$]. Our main objective was to use ovule culture/embryo rescue to overcome this fertility barrier; secondary objectives were to determine if pairing would occur between the $(AD)_1$ and $C_1$ genomes and if the glanded-plant/glandless-seed trait would be expressed in other backcross generations. Pollination of $BC_1$ plants in the field followed by ovule culture/embryo rescue produced 14 mature $BC_2$ progeny with partial female fertility, demonstrating that tissue culture circumvented fertility barriers of the pentaploid interspecific hybrids. One $BC_2$ had no glands while in culture, but was fully glanded after the first true leaf emerged. From this "glandless-seed" plant, eight $BC_3$ plants [$2n = 55$ or 57] and 125 $BC_4$ plants have been derived. Five $BC_4$ seed were completely glandless. Occurrence of trivalent chromosome associations indicated $C_1$ chromatin was transferred in the backcross generations. The average rate of AAC and/or DDC trivalent formation was 5.16% for $C_1$ chromosomes of $BC_1$ to $BC_4$ plants. Germplasm derived from this material could be important for developing cotton with higher levels of insect resistance and/or improved nutritional use.**


*Additional index words:* **Interspecific hybrids, Gossypol, Terpenoids, Tissue culture, Embryo rescue, Genomes, Homoeologues, *Gossypium hirsutum* L.**

PROTEIN products and oil from cottonseed (*Gossypium* spp.) have restricted use and additional processing requirements because of the presence of a sesquiterpenoid, gossypol, in lysigenous glands in the embryo (4). Certain genetic mutations at the $Gl_2$ and $Gl_3$ loci of cultivated upland cotton, *Gossypium hirsutum* L. [$2n = 4x = 52$; genomic formula $2(AD)_1$], eliminate glands from all plant parts, including seed. However, completely glandless genotypes are more susceptible to phytophagous insects in nursery tests (e.g., 8) and have been predicted to have limited production potential (11). Higher gossypol content in leaves or flowerbuds is correlated with improved resistance to tobacco budworm and bollworm (*Heliothis*) (2). A principal deterrent to using high-gossypol germplasm for insect control is that gossypol levels in seed are concomitantly increased, limiting cottonseed utilization. Combined agricultural and seed processing needs would be satisfactorily met by developing *G. hirsutum* genotypes that form glanded aerial parts and glandless seed. A cotton plant with this attribute could also be extremely useful for developing insect-resistant cotton with higher gossypol.

Plants of several wild Australian diploid species, including *Gossypium sturtianum* Willis [$2n = 2x = 26$; $C_1$ genome], are glanded but their seed are functionally glandless and do not contain gossypol (7). Hexaploid populations of *G. hirsutum* × *G. sturtianum* have been developed by Muramoto (10). An off-type plant in one of these interspecific hexaploid populations [presumably $2n = 6x = 78$; genomic formula $2[(AD)_1C_1]$] was identified by Dilday as having a glanding pattern similar to *G. sturtianum* (6). Putative pentaploids [$2n = 5x = 65$; genomic formula $2(AD)_1C_1$] derived by backcrossing a hexaploid reportedly expressed the glanded-plant and glandless-seed trait and were fertile, but were not analyzed cytogenetically (6). This *G. hirsutum* × *G. sturtianum* germplasm was part of our research unit's experimental materials, and all first backcross ($BC_1$) plants, presumably pentaploid, were determined to be sterile in both greenhouse and field environments based on 4 yr of attempted self-pollination and cross-pollination.

Fertile hexaploids from *G. hirsutum* × *G. sturtianum* hybrids were reported previously (1, 3, 10), but further introgression of $C_1$ genome chromatin with this population appeared to be unlikely by conventional means. Crossing barriers at the pentaploid level have been recognized for many interspecific hybrids of *Gossypium* such as those involving the $B_1$ and $E_1$ genomes (14). Volkova (14) reported that only 5 to 13% of meiocytes from these pentaploids contained 26 bivalents and 13 univalents, and that numerous anomalies typically causing sterility, such as multivalents, bridges, and polyads, were observed.

The feasibility of successfully introgressing any trait from *G. sturtianum* has been doubted because of the infrequency of pairing between $C_1$ and $(AD)_1$ chromosomes. Brown and Menzel (3) reported $2[(AD)_1C_1]$ hexaploids had among the lowest number of irregular associations at metaphase of the 16 allohexaploids that were cited. Multivalent frequencies were lower only for the hexaploid $2[(AD)_1E_1]$. Louant et al. (9) advocated selfing for several generations at the hexaploid level to introgress $C_1$ germplasm, based on the assumption that pairing frequencies in the $F_1$ hexaploids, as reported by Menzel and Brown (3), inaccurately predict potentials for intergenomic recombination. Da Silva et al. (5) confirmed the observations of Brown and Menzel (3) and also analyzed the hexaploid $F_1$, $F_2$, and $F_3$ generations. Although heterogenetic associations occurred, these latter researchers concluded that selfing did not increase recombination potential of the hexaploids and that C-genome transfer to *G. hirsutum* would be extremely difficult.

Techniques for ovule culture and embryo rescue have been defined for *Gossypium* (12). This type of tissue

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culture has been successfully utilized for recovering $F_1$ interspecific cotton hybrids (13), but there has been no report of the applicability of these techniques for overcoming other fertility barriers. The primary objective of this research was to determine if ovule culture could be used to overcome substantial fertility barriers in early stages of $C_1$ genome introgression. Secondly, assuming progeny beyond $BC_1$ could be derived with tissue culture, our objective was to ascertain if genome recombination occurred and if the glanded-plant and glandless-seed trait would still be observed.

## MATERIALS AND METHODS

Plants of this study designated as hexaploids and $BC_1$'s to *G. hirsutum* cv. TM-1 were those previously reported from *G. hirsutum* × *G. sturtianum* hybrids by Dilday (6). Seed were produced by the hexaploid plants, but none were produced in vivo by $BC_1$ plants grown in Brownsville and College Station, TX. Selected $BC_1$ plants were used as females in crosses with *G. hirsutum* cv. CAMDE, and modified procedures of Stewart (12) for ovule culture and embryo rescue were used to produce $BC_2$ progeny.

Ovules were aseptically excised 2 days after fertilization and floated on 15 mL of medium in 25- × 150-mm culture tubes. The ovule culture medium contained in milligrams per liter: 272.180 $KH_2PO_4$, 6.183 $H_3BO_3$, 0.242 $Na_2MoO_4 \cdot 2H_2O$, 441.060 $CaCl_2 \cdot 2H_2O$, 0.833 KI, 0.024 $CoCl_2 \cdot 6H_2O$, 493.000 $MgSO_4 \cdot 7H_2O$, 16.902 $MnSO_4 \cdot H_2O$, 8.627 $ZnSO_4 \cdot 7H_2O$, 0.025 $CuSO_4 \cdot 5H_2O$, 535.000 $NH_4Cl$, 8.341 $FeSO_4 \cdot 7H_2O$, 11.167 $Na_2EDTA$, 5055.500 $KNO_3$, 0.492 nicotinic acid, 0.822 pyridoxine HCl, 1.349 thiamine HCl, and 180.160 myo-inositol. The ovule medium was adjusted to pH 7, supplemented with 4% sucrose (w/v), and autoclaved (18 min, 125°C, and 138 × $10^3$ Pa). Ovules were cultured at 30°C in the absence of light for 28 to 56 days.

Embryos were removed under sterile conditions and placed onto 35 mL of solidified medium in 250-mL Erlenmeyer culture flasks. Embryo culture medium was composed of the following in milligrams per liter: 505.000 $KNO_3$, 240.000 $NH_4NO_3$, 492.600 $MgSO_4 \cdot 7H_2O$, 175.800 $CaCl_2 \cdot 2H_2O$, 27.200 $KH_2PO_4$, 1.830 FeEDTA, 5.070 $MnSO_4 \cdot H_2O$, 2.590 $ZnSO_4 \cdot 7H_2O$, 0.249 KI, 0.217 $Na_2MoO_4 \cdot 2H_2O$, 0.008 $CuSO_4 \cdot 5H_2O$, 0.007 $CoCl_2 \cdot 6H_2O$, 0.492 nicotinic acid, 0.822 pyridoxine HCl, 1.349 thiamine HCl, 0.200 gibberellic acid ($GA_3$), and 0.200 naphthaleneacetic acid. After supplementing the embryo medium with 2% sucrose and 0.8% agar (w/v) and adjusting to pH 7, the medium was autoclaved as indicated above. Germinating embryos were incubated at 30°C with a 16-h photoperiod (400 $\mu$mol $m^{-2}$ $s^{-1}$). Generally, after the plantlets had reached the second true-leaf stage, they could be removed for hardening.

Hardening was accomplished by placing the plantlets in a distilled water ($dH_2O$)–saturated, sterilized soil mix of peat:sand:vermiculite (1:2:1) supplemented with dolomitic lime and Osmocote[3] slow-release nutrients. Plastic cups (Solo[3] 16 oz.) with drainage holes were used as containers and were covered with a perforated plastic tent supported by 230-mm wooden garden stakes. Plants were incubated at 25°C and continuous light (250 $\mu$mol $m^{-2}$ $s^{-1}$) for 14 days, with the plastic tent removed after the seventh day. Following hardening, the surviving plants were grown in the greenhouse or field in the identical manner as other cotton germplasm.

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA, and does not imply its approval to the exclusion of other products that may also be suitable.

Because the gene(s) for the absence of seed glanding in *G. sturtianum* were expressed in seed embryos, gland expression for all embryos was rated as: (I) glandless, (II) very reduced glands confined to cotyledonary edges, (III) reduced glands throughout the embryo, and (IV) normal glands. Ovule-culture plants were observed throughout embryo germination. Seed glandedness was rated following imbibition of half-strength Hoagland's solution from saturated germination paper for 4 to 6 h in the dark at 30°C, removal of seed coats, and examination of the embryos with a Zeiss SR Stereomicroscope.[3]

All $BC_2$ plants were recovered via ovule culture and embryo rescue, but subsequent $BC_3$ and $BC_4$ plants were obtained with standard hybridization techniques, except that all pollinated flowers were treated with several drops of 500 mg $GA_3$ $L^{-1}$ in $dH_2O$. The male parent was *G. hirsutum* cv. CAMDE.

Flowerbuds were fixed in 95% ethanol:glacial acetic acid (3:1) for cytogenetic analysis. Standard acetocarmine stain was used to prepare microsporocyte squashes; preparations were examined with a Zeiss Universal[3] compound microscope. All $BC_2$, $BC_3$, and $BC_4$ plants and those hexaploids and $BC_1$ plants used for cytogenetic analyses were grown in the nursery or greenhouses at College Station from 1983 to 1986. Additionally, glandless-seeded hexaploids and unselected *G. hirsutum* × *G. sturtianum* hexaploids were grown and scored for glands.

## RESULTS AND DISCUSSION

Cytogenetic analyses of a subset of the hexaploid and $BC_1$ germplasm selected by Dilday (6) confirmed their putative ploidy (Fig. 1 and 2). Initial efforts at ovule culture yielded $BC_2$ progeny from each of two $BC_1$ pentaploids. One of these $BC_2$ plantlets lacked glands in the cotyledons, hypocotyl, and radicle (Category I), but had fully glanded parts after the first true-leaf stage. All further ovule culture was with ovules produced after pollinating flowers of the two productive $BC_1$ pentaploids and their $BC_2$ progeny.

Approximately 10 000 ovules were cultured with 1% embryo recovery. Umbeck and Stewart (13) reported embryo production ranging from 0 to 18.2% in interspecific crosses. These authors also reported that embryo rescue should generally be completed by 25 days postanthesis or severe embryo atrophy could occur. We observed that most of the excised embryos from developed ovules had atrophied (and were not counted for embryo production data), and a higher recovery rate might have been possible by excising embryos at an earlier stage. Fourteen mature $BC_2$ plants were obtained, and cytogenetic analyses confirmed a reduction in the number of $C_1$ chromosomes (Fig. 3).

Most individual $BC_2$ plants had from 6 to 10 $C_1$ chromosomes and a normal complement of 26 $(AD)_1$ bivalents. The Category I $BC_2$ plant had eight $C_1$ chromosomes. None of the backcross plants exhibited normal glanding (Category IV), and glands were lighter in pigmentation than typical gossypol glands of *G. hirsutum*. Five of the $BC_2$ plants had very reduced glands confined to cotyledonary edges (Category II), and eight had reduced glands throughout the embryo (Category III).

No $BC_3$ progeny were obtained by ovule culture, but seed were obtained from $GA_3$-treated, pollinated flow-

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ers of $BC_2$ plants. A total of eight mature $BC_3$ plants were recovered, with zero, three, four, and one plants having embryo glanding patterns corresponding to Categories I through IV, respectively. These $BC_3$ plants were derived from the Category I $BC_2$ plant. All of the $BC_3$ plants had either three or five $C_1$ chromosomes, usually present as large univalents (Fig. 4). The occasional occurrence of AAC or possibly DDC chromosome trivalents indicated that intergenomic recombination was occurring at each backcross generation. Quadrivalents and larger multivalents were sometimes observed (Fig. 5). All $BC_3$ plants flowered, and 125 $BC_4$ seed were produced after one greenhouse season. There were 5, 43, 59, and 18 embryos rated in Categories I through IV, respectively (Fig. 6).

A total of 375 Metaphase I cells from 26 plants have been analyzed from the $BC_1$ to $BC_4$ generations. Of the 2382 $C_1$ chromosomes observed, 123 were associated as trivalents with A or D chromosomes, whereas the remainder were present as nonassociated, large univalents (Fig. 2, 3, and 4). Although larger multivalents were occasionally observed (Fig. 5), these could not be definitely determined as including $C_1$ chromatin. The trivalent data indicated that 5.16% or about one out of every 20 $C_1$ chromosomes was associated with $(AD)_1$ chromosomes at Metaphase I. Because previous reports (1, 3, 5, 6, 9, 14) do not give detailed cytogenetic data other than for hexaploids from *G. hirsutum* × *G. sturtianum* hybrids, there are no comparable pairing data for evaluating our results. Brown and Menzel (3) did give trivalent frequencies for six other pentaploids, and the lowest value was 1.79 per cell for $2(AD)_1B_1$. We observed an average of 1.22 trivalents per cell in 59 Metaphase I meiocytes from pentaploid $BC_1$ plants $[2(AD)_1C_1]$, so the level of pairing was relatively low. However, the observation that $C_1$ associations continued through the $BC_4$ generation indicated that some recombination occurred, which should allow for $C_1$ introgression. Whether the pairing was due to recombination during selfing of hexaploid populations, as proposed by Louant et al. (9), or rep-

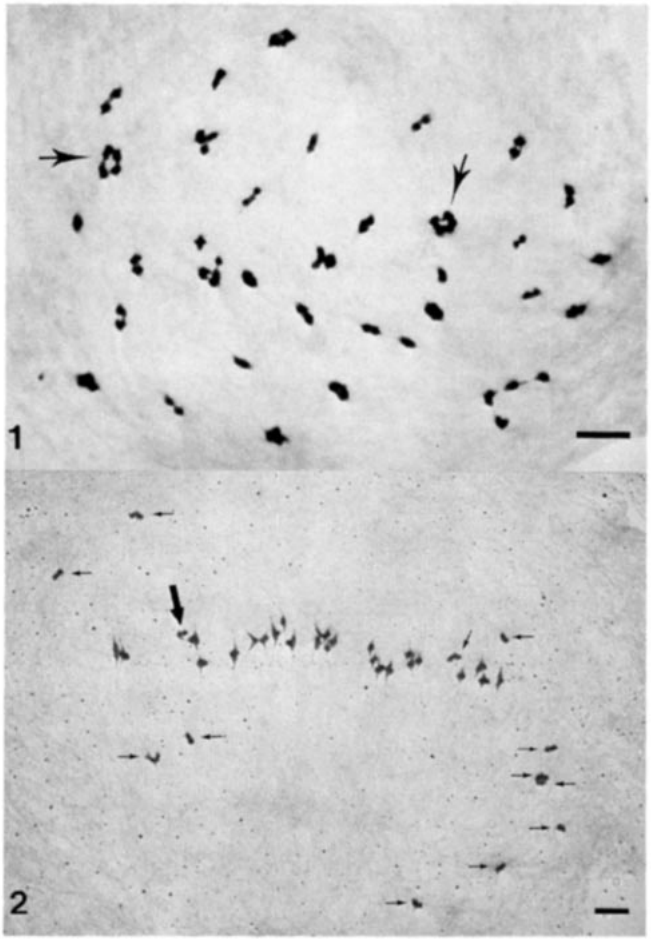


**Fig. 1–2.** ***Fig. 1.*** **Metaphase I spread of a microsporocyte from a hexaploid $[2n = 6x = 78 = 2[(AD)_1C_1]]$ derived from *G. hirsutum* $[2n = 4x = 52 = 2(AD)_1]$ × *G. sturtianum* $[2n = 2x = 26 = 2C_1]$ hybrid; 35 bivalents and two quadrivalents (arrows) with possibly additional multivalent associations, e.g., chain of four in lower right. Bar represents 10 μm.** ***Fig. 2.*** **Metaphase I spread of a microsporocyte from a $BC_1$ pentaploid $[2n = 5x = 65 = 2(AD)_1C_1]$ derived from a *G. hirsutum* × *G. sturtianum* hexaploid $[2n = 6x = 78 = 2[(AD)_1C_1]]$; 25 bivalents, 12 univalents (small arrows), and one trivalent (large arrow). Bar represents 10 μm.**

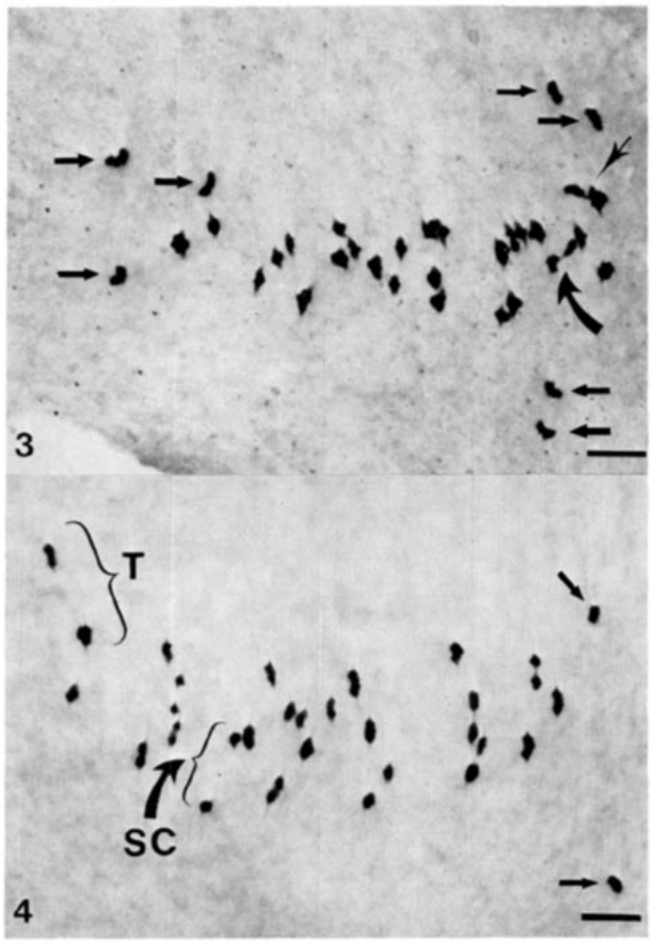


**Fig. 3–4.** ***Fig. 3.*** **Metaphase I spread of a microsporocyte from a $BC_2$ aneuploid $[2n = 4x + 8 = 60 = 2(AD)_1 + 8\ C_1$-chromosomes] derived by ovule culture from a *G. hirsutum* × *G. sturtianum* $BC_1$ pentaploid $[2n = 5x = 65 = 2(AD)_1C_1]$; 24 typical bivalents, one single chiasma bivalent (curved arrow), seven univalents (thick arrows), and one trivalent (attenuated arrow). Bar represents 10 μm.** ***Fig. 4.*** **Metaphase I spread of a microsporocyte from a $BC_3$ aneuploid $[2n = 4x + 3 = 55 = 2(AD)_1 + 3\ C_1$-chromosomes] derived from a *G. hirsutum* × *G. sturtianum* $BC_2$ aneuploid $[2n = 4x + 8 = 60 = 2(AD)_1 + 8\ C_1$-chromosomes]; 25 typical bivalents, one single chiasma bivalent partly disjoined (SC), two univalents (small arrows), and one trivalent (T). Bar represents 10 μm.**

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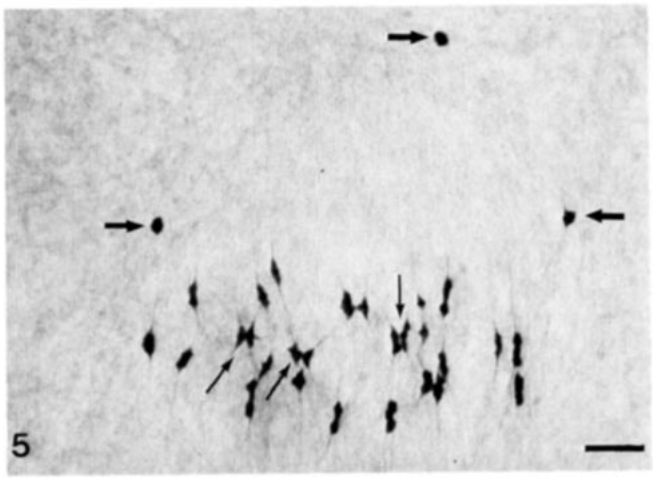


**Fig. 5. Metaphase I spread of a microsporocyte from a $BC_3$ aneuploid [$2n = 4x + 3 = 55 = 2(AD)_1 + 3\ C_1$-chromosomes] derived from a *G. hirsutum* × *G. sturtianum* $BC_2$ aneuploid [$2n = 4x + 8 = 60 = 2(AD)_1 + 8\ C_1$-chromosomes]; three to five multivalent associations with three definite quadrivalents (attenuated arrows) and three univalents (thick arrows). Bar represents 10 μm.**

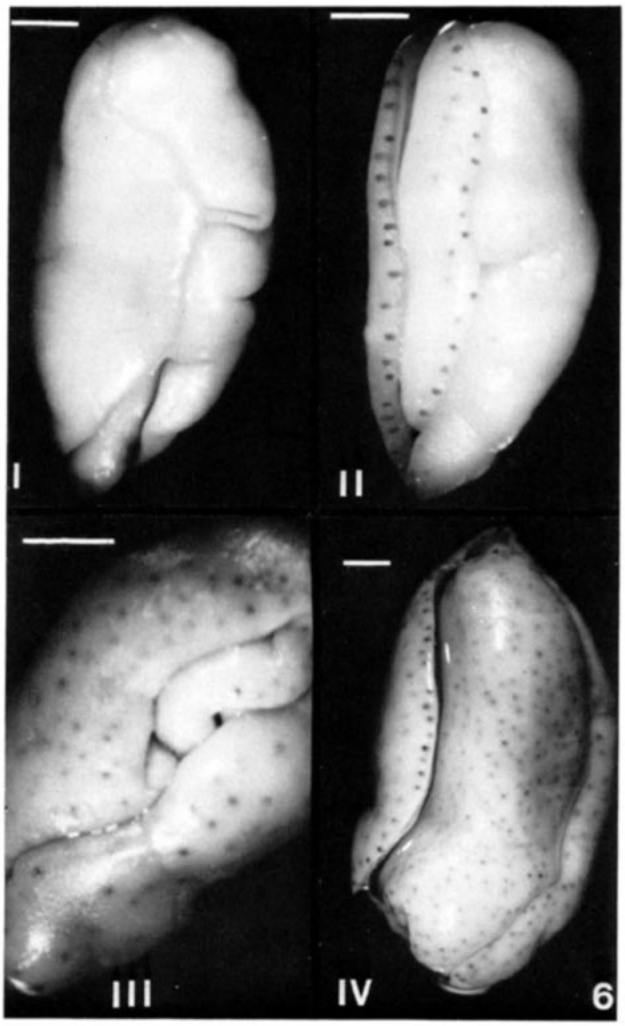


**Fig. 6. Gossypol glanding patterns in seed from *G. hirsutum* × *G. sturtianum* fourth backcross with the recurrent parent *G. hirsutum* cv. CAMDE; I, II, III, and IV correspond to glanding categories of (I) glandless, (II) very reduced glands confined to cotyledonary edges, (III) reduced glands throughout the embryo, and (IV) normal glands, respectively. Bar represents 1 mm.**

resents the inherent recombination potential of the $C_1$ genome cannot be determined.

As might be anticipated from wide interspecific hybridization, many morphological characters were extremely variable among the backcross progenies (Fig. 7, 8, and 9). Floral parts approached the *G. hirsutum* type with increasing number of backcrosses (Fig. 7). Bolls (Fig. 8) and growth habit (Fig. 9) were especially aberrant although several aneuploids had characteristics that resembled typical tetraploid cotton. The occurrence of reduced glands in the seed with normal glanding on other plant parts is unique for interspecific germplasm with primarily a *G. hirsutum* background.

Recovery of a 52-chromosome *G. hirsutum* pure line expressing the glandless seed trait of *G. sturtianum* is not certain from our data. Scrutiny of selfed seed from

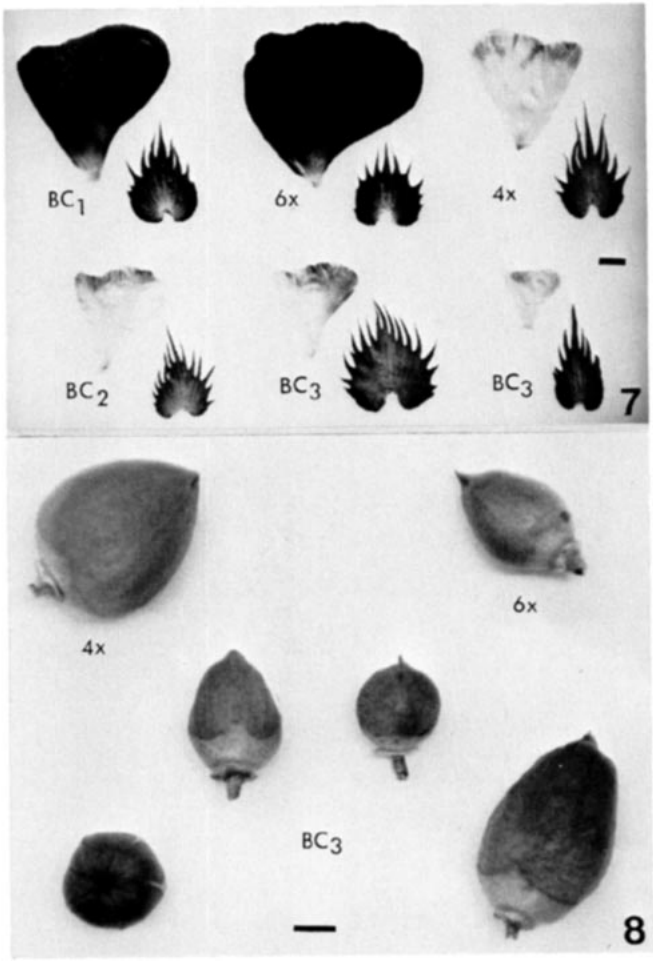


**Fig. 7–8. *Fig. 7.* Petals and floral bracts from *G. hirsutum* × *G. sturtianum* plants with varying number of backcrosses to *G. hirsutum*; $4x$ = *G. hirsutum* cv. CAMDE [$2(AD)_1$], $6x$ = hexaploid {$2[(AD)_1C_1]$}, $BC_1$ = first backcross progeny from hexaploid [$2(AD)_1C_1$], $BC_2$ = second backcross progeny from hexaploid obtained via ovule culture [$2(AD)_1 + 8\ C_1$-chromosomes], and $BC_3$ = third backcross progeny from hexaploid [from left to right: 51 $(AD)_1$-chromosomes + 5 $C_1$-chromosomes and $2(AD)_1 + 5\ C_1$-chromosomes, respectively]. Bar represents 1 cm. *Fig. 8. Gossypium hirsutum* × *G. sturtianum* boll morphology from four third backcross progeny [$BC_3$; genomic formula from left clockwise: $2(AD)_1 + 5\ C_1$-chromosomes, $2(AD)_1 + 3\ C_1$-chromosomes, $2(AD)_1 + 5\ C_1$-chromosomes, and 51 $(AD)_1$-chromosomes + 5 $C_1$-chromosomes, respectively] from the hexaploid {$6x$; $2[(AD)_1C_1]$} with the recurrent parent *G. hirsutum* cv. CAMDE [$4x$; $2(AD)_1$]. Bar represents 1 cm.**

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**Fig. 9. Ovule culture derived *G. hirsutum* × *G. sturtianum* second backcross plant (recurrent parent: *G. hirsutum*) with aberrant growth habit including shortened internodes, abnormal leaves, and the absence of fruiting parts. Bar represents 5 cm.**

the original glandless-seeded plants and unselected hexaploids indicated that expression of glandedness is variable. Backcross embryos derived through ovule culture or conventional hybridization have been smaller than those of *G. hirsutum*. Cotyledons of some of this germplasm, especially of embryos from ovule culture, were malformed and reduced in size. Because we did not identify a hexaploid that consistently produced completely glandless seed and the backcross embryos were atypical, modification of trait expression cannot be ruled out. The degree to which developmental and/or environmental factors affect seed glandedness in this genetic background is not known. Cytogenetic verification of $C_1$-chromosome associations with $(AD)_1$ chromosomes demonstrated the feasibility of introgressing genes from *G. sturtianum*. Glandless and reduced-glanded progeny, Categories I, II, and III, could be expressing introgressed gene(s).

This experimental germplasm may have several additional uses that could be important, besides specific incorporation of the *G. sturtianum* seed trait into *G. hirsutum*. Although genetic diversity and germplasm utilization have been frequently advocated for major crop species, detailed basic studies of the genetics and cytogenetics for specific trait introgression with cotton have rarely been conducted. *Gossypium* has a perennial growth habit that has allowed the preservation of all plants from this ovule culture program. These plants could be used to study exotic-germplasm introgression, particularly in regard to pairing and recombination with different selection schemes.

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