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Substitution of Cotton Cytoplasms from Wild Diploid Species for Cotton Germplasm Improvement[1]

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ABSTRACT

The purpose of this investigation was to initiate a program of cytoplasm introgression from the wild diploid species into cultivated cotton (*Gossypium hirsutum* L.) and then use the semigametic trait of Pima cotton (*G. barbadense* L.) to develop homozygous cytoplasmic-substituted lines for evaluation and commercial release. It was determined that the in situ pattern of ovule and embryo growth in the wild species is similar to the cultigens but that the wild relatives have a shortened period of boll development, show reduced seed size and weight, have fewer seeds per boll, and in some cases require specific light conditions in order to initiate flowering. Three new interspecific hybrids with wild species cytoplasms were recovered by using four different pollen parent sources, a single application of GA_3 at 3.5 mmol L^{-1} to the flower at anthesis, and embryo rescue techniques. Results indicated that the success of fertilization and embryo development was strongly influenced by the paternal species used to make the cross, and secondly, that the degree of hybrid embryo development may be a more important factor than the age or size at the time of embryo rescue. Immature embryos rescued prior to 15 days post-anthesis failed to show further development or undergo precocious germination. The optimum time for embryo rescue and recovery occurred between 15 and 25 days after anthesis. Cytogenetic analysis of the hybrids verified that they were true interspecies crosses. Observations of meiotic metaphase chromosomes indicated the degree of relatedness between species. As expected, chromosome pairing indicated a very close homoeology between the *G. trilobum* DC. ex Skov. (D_8) genome and the D subgenome of *G. hirsutum*. The E genomes of *G. stocksii* Mast ex Hook. (E_1) and *G. somalense* (Gürke) Mayer (E_2) exhibited little homoeology to either the A or the D subgenome of *G. hirsutum* or the A genome of *G. arboreum* L. Cytogenetic observations by others on the reciprocal hybrids of these species closely agree with our data on chromosome associations. Thus, there does not appear to be a significant effect of cytoplasm on chromosome pairing in the primary hybrids.

Additional index words: **Interspecific hybridization, Embryo rescue, Cytogenetics.**

THE GENETIC improvement of crops requires the assimilation of germplasm resources which can then be advanced into breeding materials. In order to accomplish this objective one approach has been to make interspecific hybrids but because of different chromosome ploidy levels, individual chromosome differences, cytoplasmic differences, endosperm breakdown and lethal gene combinations, direct species hybridization has not always been successful (for review see Stalker, 1980). In cotton (*Gossypium hirsutum* L.), novel methods have been developed and used in order to bypass some of these barriers to interspecific hybridization. In the 1930s and 1940s, embryo culture was recognized as a method of recovering hybrids unobtainable by conventional breeding techniques (Skovsted, 1935; and Beasley, 1940). Because of inadequacies in the synthetic medium, embryo culture was of limited success (Lofland, 1950). Later refinements in the embryo tissue culture medium by Mauney (1961) showed that immature embryos of cultivated cotton could be isolated and grown to maturity in an artificial environment. Despite these improvements no one reported using embryo rescue as a method of recovering rare interspecific hybrids. An important breakthrough came with the development of a cotton ovule culture medium (Joshi and Johri, 1972; Eid et al., 1973; and Beasley et al., 1974). Further medium improvements by Stewart and Hsu (1977) produced a convenient and reliable method of ovule-embryo culture which resulted in the complete recovery of mature cotton plants. Recent applications have focused on the recovery of interspecific hybrids between cotton tetraploid cultivars and their diploid wild relatives (Stewart and Hsu, 1978; and Stewart, 1979)

Most cotton interspecific hybrids have been derived from crosses between cultivated cottons as the maternal parent and wild species or just wild species crosses (Beasley, 1940; and Anonymous, 1981). Only marginal success had been obtained where the wild relative functioned as the maternal parent. Since Meyer's (1973) reported development of commercial cottons with three wild species cytoplasms from *Gos-*

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sypium anomalum Wawr. and Peyr., *G. harknessii* Brandg. and *G. longicalyx* Hutch. and Lee, no other diploid cytoplasms have been captured and introgressed into commercial cotton. It appeared that by using ovule culture or embryo rescue one could greatly facilitate the recovery of new wild species cytoplasms and introgress these into commercial cultivars.

This investigation reports the successful interspecific hybridization between three additional wild species as maternal parents and cultivated cotton. The approaches used to overcome species incompatibility were the application of an exogenous hormone at anthesis to prevent premature boll abscission, the use of a diverse group of breeding lines as paternal parents, and the employment of ovule and embryo rescue techniques to recover mature hybrid plants.

MATERIALS AND METHODS

Plant Materials

Naturally occurring diploid, 2n=26, cotton species used as female parents were *Gossypium trilobum* (DC.) Skov. (genome D_8) *G. stocksii* Mast. ex Hook. (genome E_1) and *G. somalense* (Gürke) Hutch (genome E_2). The male parents were *G. hirsutum* L. (genome $(AD)_1$), a tetraploid, 2n=52, glandless breeding line designated Acala G.8160; *G. barbadense* L. (genome $(AD)_2$), a tetraploid glandless breeding line designated Pima 76-4050; *G. herbaceum* L. (genome A_1), a diploid breeding line designated CB2486; and the diploid *G. arboreum* L. (genome A_2) 'Nanking'. All plant materials were maintained under greenhouse conditions.

Ovule and Embryo Growth Measurements

Plants, bolls, and ovules were randomly selected from greenhouse materials. At 5-day intervals after self-fertilization in the wild species and at 10-day intervals for the cultivated lines, delinted ovule and excised embryo lengths were measured using a dissecting microscope fitted with a micrometer ocular lens. Individual embryo fresh weights were recorded on an electrobalance (Cahn #28).[3] Individual measurements were continued until the time of boll ripening (opening). Each data point is an average of twelve measurements.

Emasculation and Pollination

Flowers on the maternal parent species were emasculated one day prior to anthesis and protected from contamination by enclosure in glassine bags (Lee, 1980). At anthesis, flowers were hand pollinated with a cultivated line and treated with the plant growth hormone, gibberellic acid (GA_3), at a concentration of 3.5 mmol L^{-1}. One drop of the hormone solution was placed at the base of the calyx in the floral cup. Flowers were resealed in glassine bags to prevent any further exposure to foreign pollen. Bolls were allowed to develop a minimum of 15 days after anthesis before examination of embryo growth.

Embryo Rescue and Culture

Immature bolls were removed and sterilized in 95% ethanol (v/v). Ovules were aseptically excised and placed into sterile petri dishes. Embryos were carefully extracted and cultured on the medium of Stewart and Hsu (1977). The low salt rooting medium was modified to exclude sucrose. Cultures were maintained on a daylight cycle of 15 h and incubation temperature between 26-30°C at 50 μmol m^{-2} sec^{-1} fluorescent illumination. Seedlings were transplanted to a sand:vermiculite:soil (2:2:1) mixture and allowed to harden-off before transfer to the greenhouse.

Cytogenetics

Pollen mother cells (PMC's) at metaphase I (M1) were prepared on slides by the standard acetocarmine anther squash technique of Menzel and Brown (1978). For each cell the number of univalents, one- and two-chiasma bivalents and the number of multivalents of each type were recorded. A total of 50 PMC's were examined for each hybrid.

RESULTS AND DISCUSSION

The in situ pattern of growth for ovules and embryos in *G. hirsutum*, *G. arboreum*, *G. stocksii*,. and *G. somalense* were determined by measuring delinted-ovule and embryo lengths and embryo fresh weight (Fig. 1). Throughout boll development ovules were easily removed for delinting and measuring. Up to 10 days after anthesis embryos were extremely small and did not show any measurable growth. Embryo growth and development was very rapid in the cultigens (Fig. 1a, b) between 10 to 30 days and in the wild species (Fig. 1c, d) between 10 to 20 days post-anthesis. During this period of development, embryo length reached a plateau but fresh weight continued to increase for approximately 10 more days. At maturation there was a slight decrease in fresh weight and a small but detectable reduction in size. As the seeds would undergo maturation and moisture loss, embryos became increasingly difficult to separate from the pericarp without damaging the embryo.

In the wild species ovule and embryo size was about one-half and fresh weight approximately one-quarter that of the cultigens. Under greenhouse conditions the average number of seeds obtained per boll for *G. hirsutum* and *G. arboreum* was 35 and 23, respectively, but for *G. stocksii* and *G. somalense* there were only 5 and 7 seeds per boll. Thus, in the wild species, the less total fresh weight gain, fewer seeds per boll and smaller seed size were obvious compensation factors for the more rapid rate of growth and development.

Other than genetic causes, embryo abortion may result from a complete lack of endosperm tissue development or an inadequate supply of the nutritive tissue to carry the embryo to full-term. Because embryos prematurely cease growth in situ, their age and size do not always coincide with their development. In general, we found that the more developed an embryo at the time of excision the greater the chance of recovery in tissue culture. For example, rescued embryos of any size lacking cotyledon development failed to germinate or show further growth. Embryos having any visible cotyledon structures would usually germinate even without continued cotyledonary development.

Based on the in situ studies of wild species embryo development it was determined that embryos isolated between 15 to 25 days post-anthesis would most likely be able to survive in culture. Our findings indicated

that hybrid embryos rescued before 15 days after fertilization would not precociously germinate or undergo further embryonic development. This result may have been due to an inadequate complement of necessary nutrients in the tissue culture medium or a species inability to survive under in vitro conditions at such a young stage of development (Lofland, 1950). The traumatic effects of excision and culture in an artificial environment also could not be ruled out as a source of detriment to immature embryo survival. In the absence of endosperm tissue formation, embryos permitted to grow beyond 25 days would begin to show signs of severe atrophy. Without embryo rescue this would lead to embryo death and seed abortion.

Frequencies of embryogenesis (Table 1) were calculated as a fraction of the total number of ovules that could potentially develop. The success of embryogenesis for each female parent was strongly influenced by the cultivated species used as a male parent. In some cases embryogenesis was detected but hybrid plants were not recovered, possibly due to lethal genomic combinations or the inability of the embryo culture medium to artificially support continued embryogenesis or precocious germination. The potential for lethal gene combinations has been characterized in some cotton interspecific hybrids. Lee (1981) demonstrated that cultivars of either *G. barbadense* or *G. hirsutum* used in combination with the wild species *G. davidsoni* Kell. will form hybrids that abort as immature embryos or young seedlings.

Gossypium hirsutum functioned as a good male par-

Table 1. The mean number of ovules per flower (± standard deviation) and the percentage of ovules producing embryos when various cultivated species are used as male parents for a particular female wild species parent. The total number of flowers pollinated is in parenthesis.

		Male parent			
Female parent	Mean no. ovules/flower	*G. hirsutum* $(AD)_1$	*G. barbadense* $(AD)_2$	*G. herbaceum* (A_1)	*G. arboreum* (A_2)
		Percentage of ovules producing embryos			
G. trilobum (D_8)	18.8 ± 2.3	3.7 (157)†	0.0 (32)	13.8 (27)	0.0 (13)
G. stocksii (E_1)	9.8 ± 1.4	13.7 (409)†	18.2 (28)	0.0 (28)	10.8 (104)†
G. somalense (E_2)	10.1 ± 2.8	1.8 (109)	9.9 (70)	0.0 (3)	15.8 (25)†

† Interspecific hybrid combinations in which at least one plant was recovered.

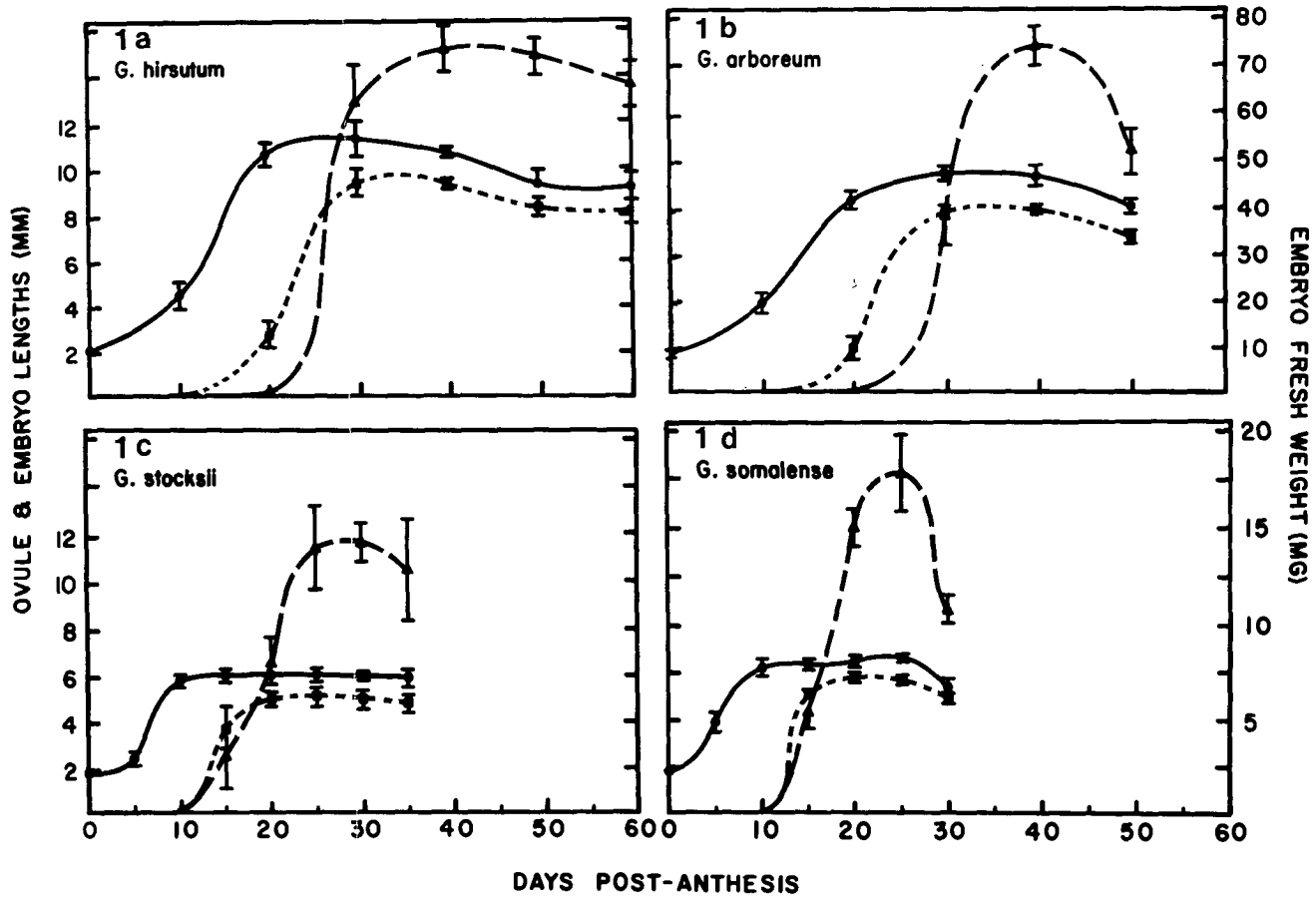

Fig. 1a–d. Species comparison of cotton ovule and embryo growth and embryo fresh weight gain in days after anthesis. ——— = ovule length, - - - - - - - - - - = embryo length and – – – – – – = embryo fresh weight. Vertical bars represent the standard deviation of 12 measurements. Note the change in scale for embryo fresh weights (right axis) between the cultigens (Fig. 1a, b) and the wild species (Fig. 1c, d).

ent with *G. trilobum* or *G. stocksii* as a female parent but failed to produce viable progeny when *G. somalense* was used as the receptive parent. The latter finding was not unexpected because we have observed that reciprocal hybrids using the same parents will die in the immature embryo to intermediate seedling stage of development. The phenomenon suggests the involvement of a lethal genomic combination in which the D subgenome of *G. hirsutum* is the source of incompatibility. Support for this observation is seen by the successful recovery of hybrids when the A species (*G. arboreum*) is used as a pollen or maternal parent with *G. somalense*. Absence of the D subgenome permitted the recovery of interspecific hybrids.

Gossypium arboreum served as a good male parent with *G. stocksii* and *G. somalense* but when crossed onto *G. trilobum* it did not produce any detectable embryos. The least successful male parents were *G. barbadense* and *G. herbaceum* and even though hybrid embryos could be detected by microscopic examination in certain combinations with these wild species, no seedlings were recovered. Thus, it was evident that there was a species effect on the success of interspecific hybridization. There are many stages and levels of difficulty to recovering interspecific hybrids. Use of the wild species as a female parent is the most difficult (Feng, 1935; and Stewart, 1981). Current technology in cotton provides few alternatives for increasing cytoplasmic diversity. In the future, it will be beneficial to examine the success of hybridization using various accessions of a particular species.

The triploid hybrids, $D_8 \times (AD)_1$ and $E_1 \times (AD)_1$, each have a total of 39 chromosomes (Table 2). Each subgenome contributes 13 chromosomes. The diploid hybrids, $E_1 \times A_2$ and $E_2 \times A_2$, have 26 chromosomes. Cytogenetic analysis of all hybrids investigated revealed no quadrivalent chromosome configurations.

The A subgenome chromosomes of *G. hirsutum* are larger relative to the D chromosomes (Skovsted,

Table 2. Summary of chromosome associations and chiasmata frequency for interspecific hybrids of cotton. Numbers in parenthesis indicate the range observed for each category.

Hybrid	Chromosome distribution per cell			Chiasmata/ bivalent
	I	II	III	
$D_8 \times (AD)_1$	12.7 (10–15)	12.1 (10–13)	0.7 (0–3)	1.98
$E_1 \times (AD)_1$	35.2 (31–39)	1.9 (0–4)	0.0	1.06
$E_1 \times (A_2)$	17.1 (5–22)	4.4 (2–9)	0.2 (0–1)	1.13
$E_2 \times (A_2)$	21.2 (14–26)	2.1 (0–6)	0.2 (0–1)	1.24

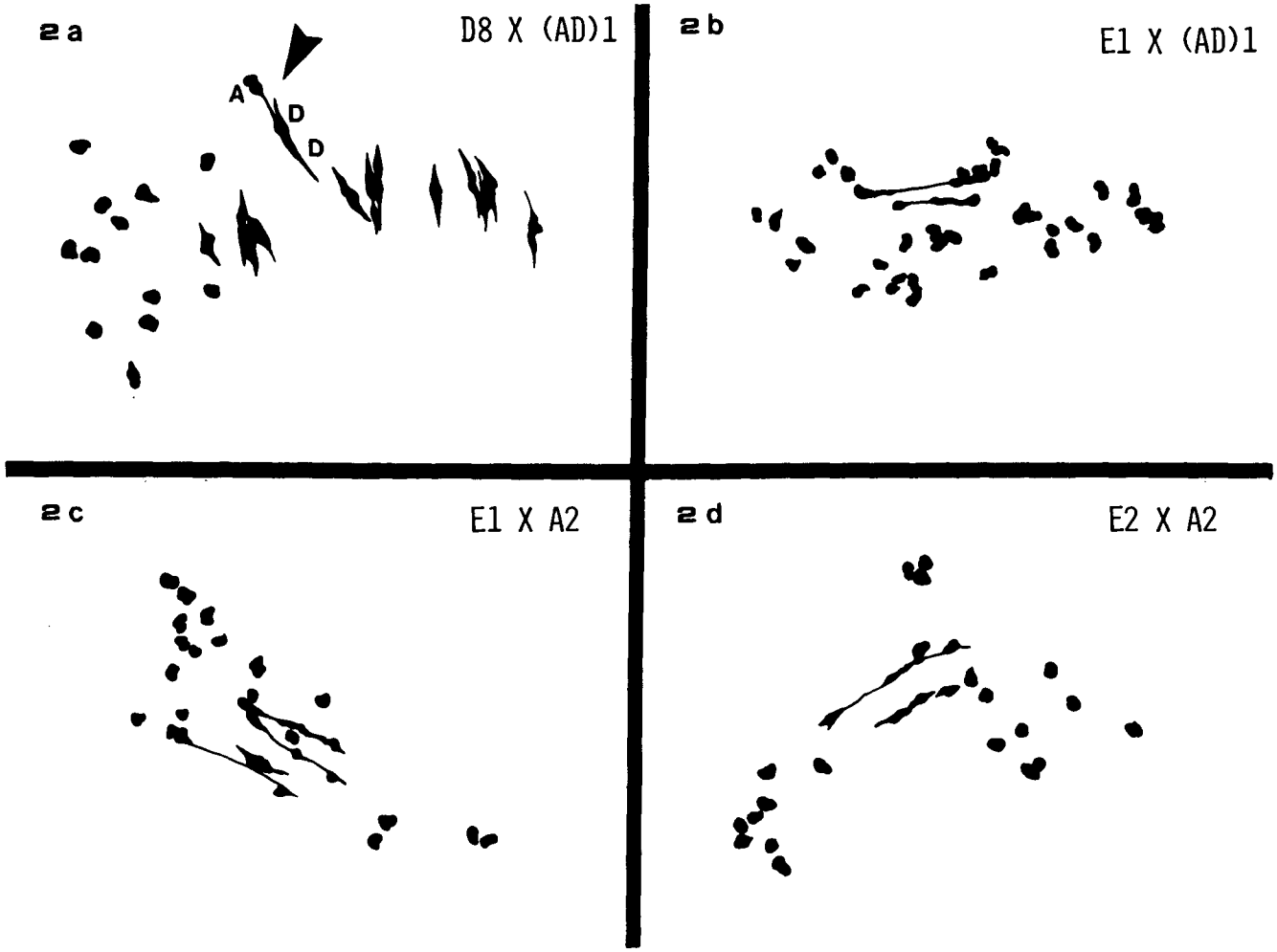

Fig. 2a–d. Metaphase I chromosome associations of cotton interspecific hybrids. A) triploid hybrid, $D_8 \times (AD)_1$, showing 12 allosyndetic bivalents (D-D subgenomic pairs), 1 trivalent (D-D-A, see arrowhead) and 12 univalents (A subgenome); B) triploid hybrid, $E_1 \times (AD)_1$, with 2 bivalents and 35 univalents; C) diploid hybrid, $E_1 \times A_2$, with 4 bivalents and 18 univalents; D) diploid hybrid, $E_2 \times A_2$, with 2 bivalents and 22 univalents.

1934). Thus a distinction may be made with respect to the type of syndetic pairing that occurs in some interspecific hybrids, especially those involving the D species. In the hybrid between *G. trilobum* (D_8) and *G. hirsutum* $(AD)_1$ almost all bivalents are allosyndetic or D-D_h pairs and show a high frequency of chiasma formation. In a few instances trivalent configurations were observed which consisted of two D chromosomes and one A chromosome (Fig. 2a arrow). Based on the high frequency of bivalent formation and chiasmata, we conclude that there is a close homoeological relationship between *G. trilobum* and the D subgenome of *G. hirsutum* (Fig. 2a). Hybrids involving the E species, *G. stocksii* (E_1) and *G. somalense* (E_2), showed little homoeology to either the A or D subgenome of *G. hirsutum* or the A genome of *G. arboreum* (Fig. 2b, c, d and Table 3). Due to the relative similarity of chromosome size between the E species and the A chromosomes, the type of syndetic pairing could not be ascertained.

Cytogenetic studies of reciprocal hybrids involving D_8, the E species and cultivated cottons show similar chromosome associations. In the hybrid $(AD)_1 \times D_8$, Phillips (1975) noted a univalent frequency of 13.44 and a chiasmata frequency of 1.76. Skovsted (1937) observed 17.3 univalents, 4.4 bivalents, and 1.07 chiasma per bivalent when E species were hybridized with A_2 and 37. 9 univalents and a chiasma frequency of 1.0 when crossed onto $(AD)_2$. Similar chromosome associations were observed in our study, indicating that cytoplasmic differences do not significantly affect chromosome pairing.

Since the development of these primary haploid interspecific hybrids, substantial progress has been made with respect to their introgression into cultivated cotton. All of the hybrids have undergone chromosome doubling using colchicine treatments. This procedure was necessary in order to restore plant fertility, and it enabled us to continue backcrossing with the cultigens. The two triploid hybrids, $D_8 \times (AD)_1$ and $E_1 \times (AD)_1$, were converted to hexaploids and have been backcrossed through the difficult pentaploid condition. The E species allodiploid hybrids became synthetic disomic tetraploid when the chromosomes were doubled.

In addition to the cytoplasms we have described in this report, three new diploid species cytoplasms have been recovered in hybrids and are now being introgressed. They include *G. capitis-viridis* Mauer (B_3), *G. armourianum* Kearn. ($D_{2\text{-}1}$) and *G. turneri* Fryx. (D_{10}). Before each of the cytoplasms are released, they will undergo a recurrent backcrossing scheme similar to the one described by Mahill (1983) using the semigametic cotton line of *G. barbadense* L. (Turcotte and Feaster, 1967 and 1974). Semigamy is a condition in cotton where, upon fertilization, the egg and sperm fail to unite. Tissues independently derived from each of these cells will be haploid and through chromosome doubling will provide homozygous lines. Hybrids with species cytoplasms are backcrossed with the semigametic, *G. barbadense* as the male, recurrent parent. Once the hybrids are homozygous semigametic, cytoplasmic substitutions for various cultivars will require only one cross. Paternally derived haploid progeny can then undergo chromosome doubling to produce fertile, disomic tetraploids carrying the new species cytoplasms.

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