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Phenotypic variation in cotton (*Gossypium hirsutum* L.) regenerated plants

Bao-Hong Zhang*,†, Qing-Lian Wang and Fang Liu***

*Key Laboratory of Cotton Genetic Improvement of the Ministry of Agriculture, Cotton Research Institute, Chinese Academy of Agricultural Sciences, Anyang Henan 455112, China
**Henan Vocation-Technical Teacher's College, Xinxiang, Henan 453003, China

Somaclonal variation could be utilized for genetic improvement of cotton (*Gossypium hirsutum* L.). Although significant progress has been made in cotton regeneration, the phenotype of regenerated plants has rarely been investigated. We report here the phenotypic variation of regenerated cotton plants. Extensive variations exist in the F_0 generation and subsequent progenies of regenerated plants. Most of the phenotypic variations in F_0-regenerated plants were physiological or epigenetic and were not inherited by offspring. However, we have obtained sterile plants, and elite lines with characters of bigger boll, higher lint percentage or longer fibre from the progenies of regenerated plants. These variations in the F_1 plants were steadily inherited into the F_2 generation. The results will promote the application of plant tissue culture to cotton improvement.

COTTON is one of the most important fibre crops. Since Davidonis and Hamilton[1] obtained the first plant regeneration via somatic embryogenesis from two-year-old callus of *Gossypium hirsutum* L. cv Coker 310, significant progress has been reported in cotton tissue culture and plant regeneration. *In vitro* cultured cells of cotton have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies[1–11]; regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root[10] and anther[11] of various cotton species. Regeneration protocols have been used to obtain genetically modified plants (insect-resistant[12,13], herbicide-resistant[13–16], disease-resistant) by *Agrobacterium*-mediated transformation[17,18] or by particle bombardment[19].

Although major progress has been made in cotton regeneration, the phenotype of regenerated plants has rarely been investigated[20,21]. We have regenerated plants from many cultivars of *G. hirsutum* L. via somatic embryogenesis[5,10,11,22–26]. This paper reports extent of phenotypic variation observed in cotton plants regenerated *in vitro*.

Seeds of *G. hirsutum* L. cvs Simian-3, CCRI 12, Sirokral 1-3 and Coker 201 were obtained from the Cotton Research Institute of the Chinese Academy of Agricultural Sciences, Anyang, China. Seed coat was completely removed, and the kernels were surface-sterilized by dipping in 0.1% mercuric chloride ($HgCl_2$) solution for 7 min. After rinsing three times with sterile water, kernels were placed on half-strength Murashige and Skoog (MS) medium[27] for germination. Hypocotyl sections (3–5 mm length) and cotyledon pieces (10 ~ 16 mm^2 surface area) from 5- to 7-day-old seedlings were placed on MS medium supplemented with 0.1 mg/l kinetin (KT), 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l indoleacetic acid (IAA) for callus induction. After 4 weeks of culture, calli were transferred to embryogenic callus induction medium (MS with 0.1 mg/l IAA and 0.1 mg/l zeatin (ZT)). After 4 to 6 weeks in this medium, embryogenic calli were transferred to the same medium for further proliferation. Every 28 days, embryogenic calli were subcultured on MS medium supplemented with 0.1 mg/l KT, 0.2 mg/l IAA and 0.1 mg/l 2,4-D.

Embryogenic calli, derived from MS medium supplemented with 0.1 mg/l KT, 0.2 mg/l IAA and 0.1 mg/l 2,4-D, were selected and transferred to MS medium supplemented with 0.1 mg/l ZT for the differentiation and maturation of somatic embryos. After 3 to 5 weeks, somatic embryos (Figure 1 *a*) at various developmental stages were observed. Mature somatic embryos, arrested at the late torpedo stage and cotyledonary stage, were selected and placed on MS medium supplemented with 0.1 mg/l ZT and 2 g/l activated charcoal for germination and plant regeneration. All media were supplemented with 30 g/l sucrose, and were solidified with 7 g/l agar (Beijing Biochemistrical Company, China). The pH of the medium was adjusted to 5.8 before autoclaving at

†For correspondence. (e-mail: zbh68@hotmail.com)

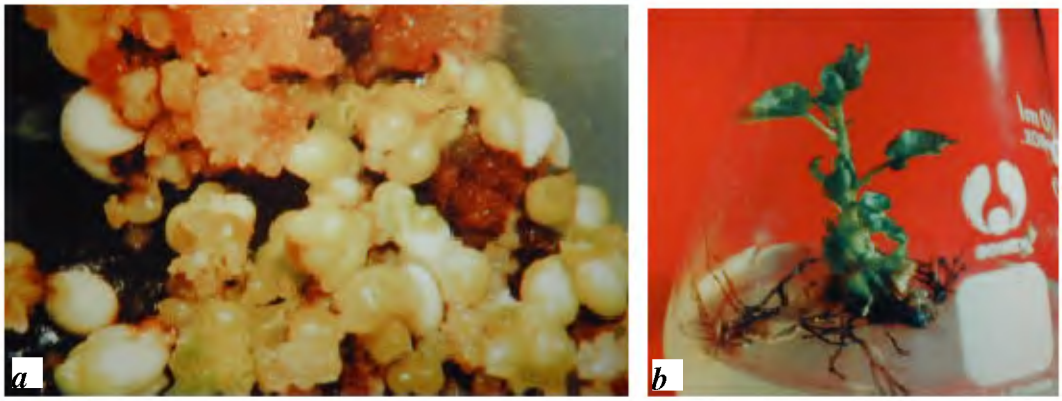

Figure 1. Somatic embryogenesis and plant regeneration in cotton. ***a***, Somatic embryos at various developmental stages; ***b***, Regenerated plant.

Table 1. Leaf shape change in F_0-regenerated plants

	Simian-3		Lumian-6		Sirokral 1–3	
Plant growth stage	Regenerated plant	Original plant	Regenerated plant	Original plant	Regenerated plant	Original plant
Early stage (0–20 days)	Round	Three-lobed	Round	Lobed	Round	Okra
Middle stage (20–40 days)	Three-lobed	Three-lobed	Three-lobed	Lobed	Three- or five-lobed	Okra
Later stage (more than 40 days)	Three-lobed	Three-lobed	Three-lobed	Lobed	Okra	Okra

121°C for 15 min. All cultures were incubated at 28 ± 2°C under a light intensity of approximately 2000 lux, provided by cool white fluorescent lamps with 16 h photoperiod.

Hardening of the regenerated plants (Figure 1 *b*) with at least three leaves was carried out in pots containing 1 : 1 (vol/vol) mixture of sterile soil and sand in the growth chamber with same condition as above. During the 2-week hardening period, regenerated plants were covered (for the first week) with a polyethylene sheet for maintaining high humidity and irrigated with progressively reduced concentrations of sterile MS medium; this was followed by irrigation with tap-water in the second week. After an additional 2 to 3 weeks of incubation, hardened plants were transferred to the greenhouse. Regenerated plants were self-pollinated or crossed with plants of the parent variety.

Extensive variations occurred in F_0 of cotton regenerated plants. The most popular variations were in the characters of plant shape, leaf shape, flower and fertility. Variation in leaf shape was the most common variation, observed in the F_0 generation of regenerated plants. No matter what the shape of parental leaves, the leaves of the F_0-regenerated plants were always round rather than their normal morphology, lobed or Okra shaped (Table 1, Figure 2 *d*). When regenerated plants were transplanted into pots and grown under natural fields conditions, the plants grew and leaf shape changed eventually to normal lobed or Okra shape (Table 1, Figure 2 *d*). Many experiments have demonstrated that cotton leaf shape is genetically controlled by gene[28] and also influenced by the developmental stage of the shoot, and endogenous levels of hormones. Leaf shape can be changed by applying GA[29]. In cotton tissue culture and plant regeneration, many hormones or plant growth regulators controlled the growth and development of regenerated plants, and influenced the hormone level of cotton plants[22]. These directly influence leaf shape and other characters of regenerated plants. With the transplantion of regenerated plants into natural condition, their endogenous hormone level would have got normalized leading to normal leaf shape.

Variations in floral organs and fertility were observed in the F_0-regenerated plants (Figure 2 *a–c*). Flowers of regenerated plants were smaller than those of the parent plants (Figure 2 *a*). Normal cotton flower has five petals, but some flowers of regenerated plants had six or seven petals, and fused-petals were also observed in regenerated plants (Figure 2 *b*). The style of regenerated plant was longer than that of the parent plant, and the stigma always projected beyond the corolla before blooming. The pedicel of the regenerated plants were shorter than those of the parent plants (Figure 2 *c*).

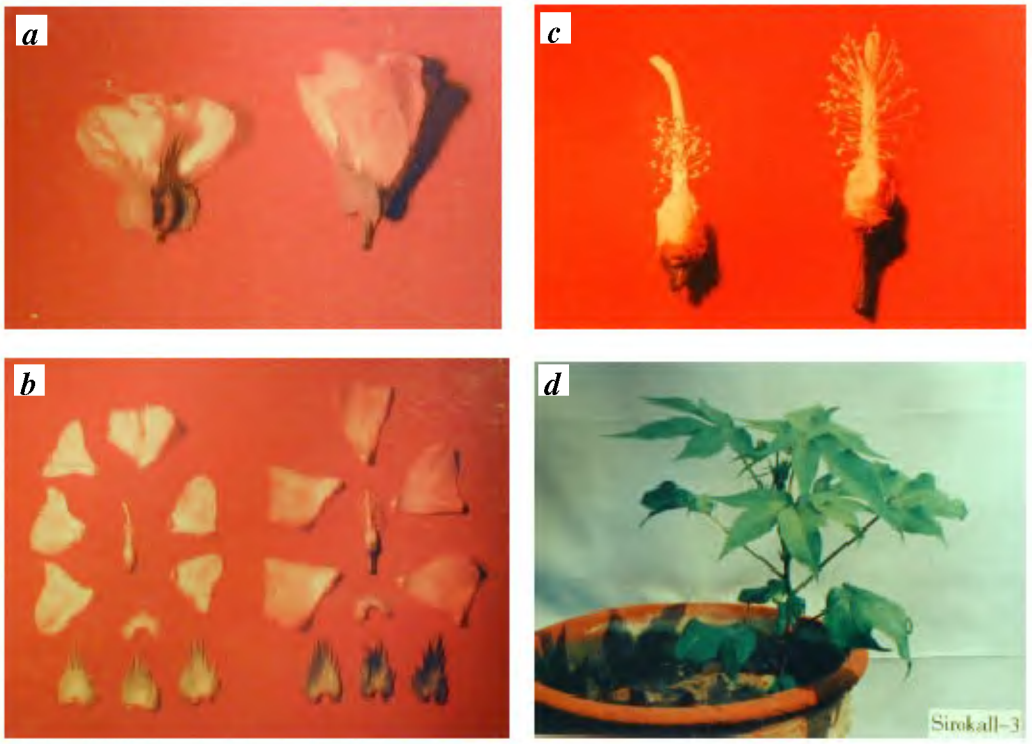

Figure 2. Phenotypic variation in cotton regenerated plants. ***a***, Flowers of regenerated plant (left) and original plant (right); ***b***, Floral parts of regenerated plant (left) and original plant (right); ***c***, Androecium and gynoecium of regenerated plant (left) and original plant (right); ***d***, Regenerated plant in plot, showing variation in leaf shape.

Table 2. Economic character variation in the F_1 progeny of cotton-regenerated plants

Variety	Character	Highest	Lowest	Range	Mean	Original parent
Lumian–6	Boll weight (g)	4.98	3.41	3.41–4.98	4.66	4.33
	Lint (%)	39.73	24.71	24.71–39.73	38.27	38.60
	Fibre length (mm)	29.15	24.04	24.04–29.15	27.98	29.20
Coker 201	Boll weight (g)	5.42	2.92	2.92–5.42	4.18	4.21
	Lint (%)	30.80	30.16	30.16–30.80	30.21	36.90
	Fibre length (mm)	29.70	27.70	27.70–29.70	27.78	27.90
Coker 312	Boll weight (g)	–	–	–	–	–
	Lint (%)	33.44	27.60	27.60–33.44	32.26	38.31
	Fibre length (mm)	30.98	29.22	29.22–30.98	30.08	29.00

Most of the F_0 plants were sterile, mainly because (1) regenerated plants had less number of anthers and quantity of pollen than original parental plants; (2) pollen development was abnormal, so that most of pollen were smaller than normal pollen and were shrivelled and irregular; (3) stigma projected before blooming. To obtain seeds, regenerated plants were pollinated with the pollen of parent plants. However, the regenerated plants turned fertile as they grew. Generally, the regenerated plants produced normal, fertile flowers after 3 months of growth in the field. However, some of the regenerated plants remained sterile after 6 months of growth; they could be due to genetic changes as the sterility could be inherited by their offspring. We have found 3 sterile plants from a population of 387 regenerated plants.

Most variations in the F_0-regenerated plants were physiological variations; they were not inherited by the offspring. Therefore regenerated plants normalized at the later growth stages of the F_0 plants or in F_1s. However, there were some genetic variations, e.g. we found 3 sterile plants in the progeny of regenerated plants (for obtaining progenies, sterile plants must be crossed with original plants); the mutation could be inherited by the offspring. Besides this, many of the variations in the F_1-regenerated plants and the subsequent progeny were

inherited. Out of these variations, some were beneficial variations.

The regenerated plants of three cultivars Lumian-6, Coker 201 and Coker 312 were selfed, which formed three somaclonal variation groups. At least 30 F_1 plants of each regenerated plant were checked. The boll weight, lint percentage and fibre length of the three somaclonal variations and their original parent plants are listed in Table 2. We clearly found variation in three characters between regenerated plants and their original plants. Mean performance of the self-crossed progenies of regenerated plants was lower than the original parent plants for the three important characters. However, some of the variations could be beneficial for breeding and practical utilization. In the progenies of Coker 201 regenerated plants, we found one plant with bolls weighing 5.42 g, which is an increase of 28.7% over the boll weight of the original parent plant group. Another plant in the progenies of Coker 312 regeneration plants showed 30.98 mm long fibres; which is 1.98 mm longer than the original parent plants (Table 2).

The characters of regenerated plants randomly changed, and several variations persisted in the regenerated plants and their offspring. Variation in the F_1 were stably inherited into the F_2 progeny and their further desired plants generations. We have self-crossed regenerated plants for 6 generations, and the variation observed in tissue culture can be inherited faithfully by offspring after the F_1 generation. From the progenies of regenerated plants, we have obtained some elite lines, with useful character of bigger boll, higher lint percentage or longer fibre.

Our results provide some evidence for the expected performance of cotton somaclones for some important agronomic traits. This information will assist both cotton breeders and scientists to make an appropriate plan for developing mutants with important characters which could be incorporated into traditional breeding programmes.

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