Theor Appl Genet (2004) 109: 472–479
DOI 10.1007/s00122-004-1663-3

ORIGINAL PAPER

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Production and characterization of somatic hybrids between upland cotton (*Gossypium hirsutum*) and wild cotton (*G. klotzschianum* Anderss) via electrofusion

Received: 27 August 2003 / Accepted: 16 March 2004 / Published online: 28 April 2004

Abstract Symmetric somatic hybrid plants between *Gossypium hirsutum* Coker 201 and *G. klotzschianum* were obtained through electrofusion. The fusion products were cultured in KM_8P medium supplemented with 2.685 μM α-naphthaleneacetic acid and 0.465 μM kinetin, and the regenerated plants were morphologically, genetically, and cytologically characterized. Nuclear-DNA flow cytometric analysis revealed that the plants tested (31 of 40) had a relative DNA content close to the total DNA contents of the two parents. Subsequent genome DNA analysis using random amplified polymorphic DNA markers revealed 16 of 18 plants were true somatic hybrids. Cytological investigation of the metaphase root-tip cells of seven hybrids revealed there were 72–81 chromosomes in the hybrids, a value close to the expected 78 chromosomes. The morphology of the hybrids was distinct from that of the parents and from that of the regenerants from protoplasts of Coker 201. Somatic hybridization represents a potent and novel tool for transferring genomes of wild cottons containing economically important traits to cultivars in breeding programs. This is the first report on the regeneration of somatic hybrids via protoplast fusion in cotton.

Introduction

Cotton is worldwide one of the most important commercial crops and consequently plays a vital role economically, politically, and socially. Chiefly a fiber crop, it has been estimated to contribute US $15–20 billion to the world's agricultural economy, with over 180 million people depending on it for their livelihood (Benedict and Altman 2001). Because cotton is highly susceptible to biotic and abiotic stresses, it requires intensive crop management. Although conventional breeding programs have made steady improvements in agronomic traits, it is becoming more and more difficult to develop new varieties. Most of the problems facing cotton production today are related to pests and diseases, and their control. Many wild species of cotton have valuable agronomic traits such as disease and insect resistance and salinity and drought tolerance. However, successful interspecific hybridization between wild species and cultivars has been limited by the necessity of embryo rescue and the low efficiency of this procedure and the low fertility of the resulting F_1 hybrids, both of which limit its use in certain breeding methods.

Recent advances in plant tissue culture technology offer novel and valuable means for cultivar improvement. Regeneration through somatic embryogenesis is preferred over organogenesis for probable single-cell origin of the somatic embryo (Merkle et al. 1995), and efficient in vitro techniques for the regeneration of plantlets from cotton are continually being developed (Firoozabady and DeBoer 1993; Kumria et al. 2003; Price and Smith 1979). Methods also have been developed for plant regeneration from cotton protoplasts (Chen et al. 1989; Finer and Smith 1982; Peeters et al. 1994).

Protoplast culture techniques have been used for many years, whereas somatic hybridization of cottons has just begun. Somatic hybridization by protoplast fusion is one method that has had a great impact on crop breeding, such as in *Brassica*, maize, wheat, *Medicago*, and rice (Cardi and Earle 1997; Liu et al. 1999; Szarka et al. 2002; Tian and Rose 1999). However, the amount of information available in these reports on successful somatic hybridization and cybridization of monocotyledons is very limited in comparison to that on dicotyledonous species. Somatic hybridization is potentially useful for the transfer of desirable traits—especially those controlled by polygenes or uncloned genes—from wild species to crop plants and for the generation of novel gene combinations by

Communicated by B. Friebe

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overcoming sexual-crossing barriers (Binsfeld et al. 2000). A unique advantage of somatic hybridization over conventional cross procedures is the possibility of exploiting new cytoplasmic combinations, which, in some cases, have led to the successful transfer of important traits such as cytoplasmic male sterility (CMS) into a new genetic background (Atanassov et al. 1998; Cardi and Earle 1997).

The aim of the investigation reported here was to determine whether it is possible to obtain somatic hybrids between *Gossypium klotzschianum* and *G. hirsutum* by means of somatic hybridization. Before protoplast fusion, the preliminary protoplast culture protocol was successfully achieved; normal fertile plants were obtained from Coker 201. Because no species-specific probes are available for cotton, we chose a simple method based on the PCR to detect the nuclear hybrids (Naoki et al. 1994; Xu et al. 1993). The nuclear composition of the hybrid plants was analyzed by flow cytometry and chromosome counting.

Materials and methods

Plant materials

Embryogenic calluses were derived from 5- to 10-mm-long hypocotyl sections of *Gossypium klotzschianum* on MSB media [MS (Murashige and Skoog 1962) medium plus B_5 vitamins (Gamborg et al. 1968)] containing 0.9 μ*M* 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 2.3 μ*M* kinetin. They were subsequently transferred onto MSB medium containing 6.8 m*M* glutamine, 3.8 m*M* asparagine, 0.25% (w/v) Phytagel (Sigma, St. Louis, Mo.), and 3% (w/v) glucose, with 0.045 μ*M* 2, 4-D, 2.5 μ*M* IBA (indole-3-butyric acid), and 0.46 μ*M* kinetin for subculturing. Embryogenic calluses of Coker 201 were induced from hypocotyl sections with 2.5 μ*M* IBA and 0.46 μ*M* kinetin. They were inoculated into 40 ml liquid MSB medium in 100-ml Erlenmeyer flasks containing 18.8 m*M* KNO_3, 13.7 m*M* glutamine, 7.6 m*M* asparagine, and 3% (w/v) glucose to establish suspension cultures for protoplast isolation. The liquid medium was removed every 7 days and replaced with 40 ml fresh liquid medium. Callus induction and protoplast culture were carried out at 28°C in either complete darkness or under light conditions [14/10-h (light/dark) photoperiod with light provided by daylight fluorescent tubes at an irradiance of approximately 33 μmol m^{-2} s^{-1}].

Isolation and fusion of protoplasts

The protoplasts of Coker 201 were isolated from the suspension cultures, and the protoplasts of *G. klotzschianum* were isolated from the embryogenic calluses, as described by Guo et al. (1998). However, a modified enzyme mixture was used: 3% (w/v) Cellulase Onozuka R-10 (Yakult Honsha, Tokyo), 1.5% (w/v) pectinase (Serva, Heidelberg, Germany), and 0.5% (w/v) hemicellulase (Sigma). The cell wall was digested for 20 h on a rotary shaker at 10 rpm. To identify protoplast viability, we stained the protoplasts with 0.048 μ*M* fluorescein diacetate (FDA) after purification. Viability was scored as the percentage of fluorescent protoplasts related to the total protoplasts at the same visual field of microscope (10×, 100–150 protoplasts/visual field, ten visual fields/sample). The protoplasts were resuspended in the electrofusion solution containing 0.50 m*M* mannitol and 0.25 m*M* $CaCl_2$ to a density of 1×10^6 ml^{-1}. The protoplasts of the two species were mixed at a 1:1 ratio. An SSH-2 somatic hybridizer (Shimadzu, Toyota, Japan) was used to mediate protoplast fusion. Approximately 1.6 ml of the mixed protoplasts was pipetted into the FTC-4 fusion chamber. The fusion procedure used was a modified version of that described by Guo et al. (1998). The mixed protoplasts were aligned with the alternate current (AC) field of 100 V cm^{-1} at a frequency of 1 MHz for 45 s. Direct current (DC), causing reversible breakdown of the aligned protoplasts, was applied six times at 0.5-s intervals at a field strength of 1,625 V cm^{-1} and a duration of 3 s to induce protoplast fusion. The protoplasts were then kept immobile for 10–15 min in order that the fusion products could regain normal shape, followed by centrifugation at 7×100 *g* for 6 min.

Protoplast culture

Following fusion, the protoplasts were cultured in the dark in KM_8P medium supplemented with 0.5 m*M* mannitol, 3% (w/v) glucose, 2.685 μ*M* NAA (α-naphthaleneacetic acid), and 0.465 μ*M* kinetin through a modified Feeder-layer culture (Assani et al. 2001) with a final density of 2.5×10^5 ml^{-1}; the embryogenic calluses of Coker 201 were used as nurse cells. Fresh KM_8P medium supplemented with 1.343 μ*M* NAA and 0.233 μ*M* kinetin was added to the culture medium at weekly intervals. The osmolality was reduced in steps of 100 mOsm kg^{-1} (by reducing the mannitol concentration) in order to reduce the mannitol level. After 6 weeks, when microcalluses had formed to a diameter of about 2 mm, the microcalluses were transferred to solid MSB supplemented with 0.054 μ*M* 2, 4-D, 0.233 μ*M* kinetin and containing 3% (w/v) glucose and 0.25% (w/v) Phytagel (pH 5.8). The light/dark regime was the same as for callus induction. Plant regeneration from calluses was carried out on MSB culture medium with 0.555 m*M* myo-inositol, 0.27% (w/v) Phytagel, 3% (w/v) glucose, and 0.05% (w/v) activated charcoal. Well-rooted plants were transferred to the greenhouse.

Flow cytometric analysis (FCA)

Approximately 1.5 cm^2 of young leaves from Coker 201, *G. klotzschianum*, and 40 of the morphologically normal fusion regenerants in vitro was chopped with a razor blade in 500 μl of an ice-cold DAPI solution (70 m*M* NaCl, 0.2 m*M* EDTA-acetic acid, 0.1 *M* Tris, 0.5% (v/v) Tween 20, 4 mg l^{-1} diamidino-2-phenylindol, pH 7.5), 1.5 ml of DAPI solution was added, and the crude samples were then filtered through a 50-μm nylon mesh. To stain the nuclei, we added 50 μl of 1 mg ml^{-1} propidium iodide solution to the filtered materials, which were analyzed using a flow cytometer (Partec, Münster, Germany).

The relative DNA content of the tetraploid Coker 201 was taken as a standard and adjusted to 100 channels; *G. klotzschianum* showed a DNA content of 39 channels. Each histogram was generated by the analysis of at least 10,000 cells and repeated four times.

Chromosome preparation

For chromosome analysis, 1-cm-long root tips were collected from seven putative fusion hybrids and their parents and pretreated in 75 m*M* KCl for 30 min. The root tips were then put into saturated *p*-dichlorobenzene for 5 h at 15°C, fixed in cold Carnoy's solution (ethanol:glacial acetic acid, 3:1) for at least 24 h at 4°C, and finally stored in 70% ethanol at 4°C. The root tips were rinsed in water and incubated in 100 μl of enzyme mixture containing 3% (w/v) cellulase Onozuka R-10, 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), and 0.5% (w/v) hemicellulase in a 10 m*M* citrate buffer, pH 4.5, for about 1.5 h at 37°C. They were then transferred to a grease-free slide, squashed in 45% acetic acid, and stained with acetocarmine. For each line, chromosomes were counted in 20 metaphase cells under a microscope.

Nuclear DNA analysis by PCR-random amplified polymorphic DNA (RAPD)

Eighteen plants from the 40 fusion plants examined by flow cytometry were randomly selected for PCR-RAPD. Genomic DNA was extracted from young leaves according to the CTAB procedure of Iqbal et al. (1997).

RAPD amplifications were carried out in a PTC-100 (Peltier Thermal Cycler, MJ Research, Waltham, Mass.) in 20-µl reaction volumes each containing 50 ng DNA, 1.8 µl 5 µ*M* primer (Sangon, Shanghai, China), 2.5 µl 10 m*M* dNTP, 2.0 µl (10× concentration) dNTP solution (Sangon), and 1 U *Taq* polymerase (Sangon). Ten 10-mer primers (S1325, S1326, S1329, S1339, S1340, S1345, S1346, S1347, S1295, and S1304; Sangon) were used for amplification of the template DNA. The sequences of S1325 and S1340 were 5′-AGTGCACACC-3′ and 5′-ACACTCGGCA-3′, respectively.

Amplification was programmed for an initial denaturation step of 94°C for 3 min, followed by 38 cycles of 94°C for 30 s, 40°C for 30 s, 72°C for 45 s, and a final extension cycle of 72°C for 10 min. On completion, 10 µl of each sample was loaded in a 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and run in TBE buffer at 4 V cm^{-1} for 3.5 h. The DNA marker used was a GeneRuler 100-bp DNA Ladder Plus (Sangon). The gels were visualized and photographed under UV light.

Results

Embryogenic callus induction and suspension culture establishment

Friable, gray-green embryogenic calluses of *G. klotzschianum* were induced from the hypocotyl sections. Following subculturing, embryogenic calluses continued to proliferate when maintained on the MSB medium supplemented with 0.045 µ*M* 2, 4-D, 2.5 µ*M* IBA, and 0.46 µ*M* kinetin. On the other hand, yellow friable embryogenic calluses were produced from hypocotyls of Coker 201 and, following their transfer into liquid medium, had uniform and concentrated cytoplasm suitable for protoplast isolation after 4 weeks.

Protoplast isolation, fusion, and culture

Protoplast yields ranged from 4×10^6 to 8×10^6 (no. protoplasts per gram fresh weight) for suspension cultures of Coker 201 and 0.8×10^6–6×10^6 (no. protoplasts per gram fresh weight) for embryogenic calluses of *G. klotzschianum*. The percentage of protoplast viability was normally higher than 90% (Fig. 1a). The percentage of fused cells reached a maximum of 70% in the most successful experiments (Fig. 1b), and the efficiency of heterokaryons reached 30%. The hybrid cells started to divide about 6 days later than the control protoplasts from suspension cultures of Coker 201 (Fig. 1c, d). The percentage of division was 31% on day 15, which is much lower than that of the controls (53%). The average plating efficiency of protoplasts was 20% in the control, while in the fused mixture it was only 1.6% (Table 1). Six weeks later, about 80 visible microcalluses had formed from fusion protoplasts. When the light-yellow microcalluses were transferred to the solid medium, embryogenic structures were produced within 3 weeks, and then plantlets were produced via somatic embryogenesis. It was very difficult to obtain normal plantlets—a large number of the embryos germinated to form abnormal plantlets without cotyledons but with strong roots. However, after 10 months, about 50 normal fusion plants had been obtained.

Morphological characteristics of the regenerated plants

The regenerated hybrid plants could be distinguished from the parental plants on the basis of morphological differences (the size and shape of leaves, the size of plant). Most of the hybrids exhibited morphological abnormalities: tufted buds and leaves (Fig. 2b–f), reduced growth, and an altered leaf shape, including needle-shaped leaves. The somatic hybrids rooted very well but had smaller leaves than the parents (Fig. 2a, h, I). None of the regenerated hybrid plants exhibited a morphology intermediate between both parents. In the growth chambers, normal plants regenerated via somatic embryogenesis from parental embryogenic calluses induced from hypocotyls, and also from protoplasts isolated from embryogenic callus. However, the hybrids showed some variations, such as reduced plant size, bulked stems, and anomalous germination.

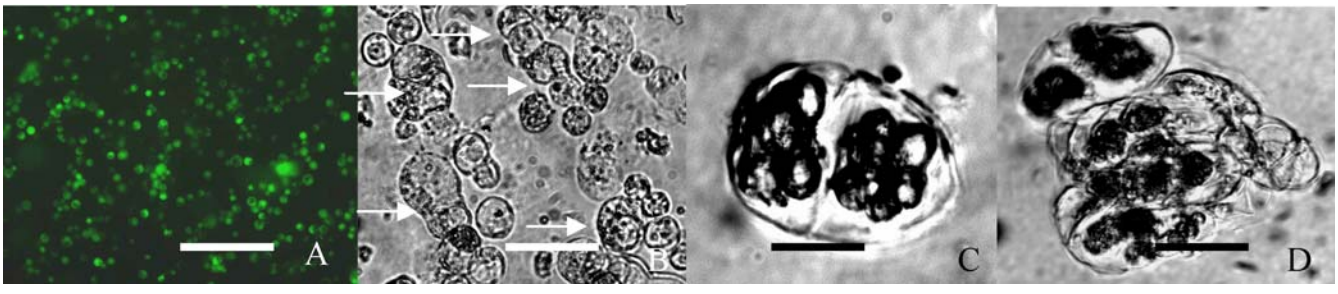

Fig. 1a–d Protoplast fusion and cell division. **a** Protoplast viability as monitored by FDA. *Bar*: 200 µm. **b** Protoplast fusion of two partners (*arrows*). *Bar*: 50 µm. **c** Cell division of the fused protoplast. *Bar*: 25 µm. **d** Cell group formation. *Bar*: 50 µm

Table 1 Plating efficiency and plant regeneration frequency in calluses derived from parental fused protoplasts and the parental unfused protoplasts

Genotype	Fusion efficiency[a] (%)	Number of protoplasts plated	Division efficiency[b] (%)	Plating efficiency[c] (%)	Number of cell masses produced
G. klotzschianum (K)		2.0×10^5	45±4.1	11±0.58	211
Coker 201 (C)		2.0×10^5	53±3.4	20±2.41	287
K+C[d]		3.5×10^5	39±2.7	13±1.03	176
K×C[e]	46±15.07	2.5×10^5	31±2.4	1.6±1.01	78

[a][Number of fused protoplasts/no. of total protoplasts in the same visual field of microscope (10×)] × 100 ± standard deviation (SD). n=6
[b](Number of protoplasts dividing/no. of total protoplasts in the same visual field of microscope) × 100 ± SD. n=6
[c](Number of protoplasts dividing and forming cell groups/no. total protoplasts in the same visual field of microscope) × 100 ± SD. n=6
[d]Mixture of the parental unfused protoplasts
[e]Fused parental protoplasts

Ploidy level

The distribution frequencies of leaf DNA contents determined using flow cytometry (Fig. 3) revealed that 31 of 40 (77.5%) normal plants obtained from hybridization were hexaploid. Using the parents' 2*x* (*G. klotzschianum*) and 4*x* (*G. hirsutum*) cotton nuclei as controls, our analysis of the hybrid regenerants showed the relative DNA contents to be the sum of those of the two parents, ranging from 132 to 135, as would be expected for the fusion between 4*x* and 2*x* protoplasts (P<0.01). Repeated analyses provided similar results. Thus, these flow cytometry profiles from young leaves represent the actual ploidy level of the plants (Miranda et al. 1997).

Chromosome analysis

Seven hybrids (detected as hexaploid by flow cytometry) were examined cytologically. Ten days following the subculture of the hybrid plantlets onto solid medium supplemented with 10 μ*M* IBA and 1.64 μ*M* kinetin, new roots of the somatic hybrids had formed easily and were collected for chromosome counting. Cells of four plants had just 78 chromosomes (52+26; Fig. 4a), as expected. Three hybrids may have been aneuploid; the chromosome number varied from 72 to 81 (Fig. 4b; P<0.01). Variation in the chromosome number in somatic hybrids is a normal phenomenon, especially in the case of spontaneous chromosome elimination in somatic hybrids between distantly related species (Binsfeld et al. 2001; Miranda et al. 1997).

Genome analysis of regenerated plants

Ten RAPD primers were used to reveal polymorphisms between the parents and the regenerated somatic hybrids. The presence of one or several specific bands of the parental species in the banding patterns of the hybrids clearly indicated the presence of the alien genome. When amplified with the S1325 and S1340 primers, the banding patterns for the parental plants Coker 201 and *G. klotzschianum* were different (Fig. 5). The presence of one or more distinct parental bands in the individual patterns of 18 plants, each of separate callus origin, confirmed their hybrid status. Primer S1325 generated multiple banding profiles in hybrid plants; three bands were present in Coker 201 (approximately 400 bp, 900 bp, and 1.4 kb) and only one band (approximately 500 bp) in *G. klotzschianum*. Some true hybrid plants (detected by flow cytometry and chromosome counting) had only partial Coker 201 bands (Fig. 5a, lanes 2, 4), indicating that they were possibly aneuploids with missing chromosomes from *G. klotzschianum* or Coker 201, or from both of the two parents. Primer S1340 produced two bands in both parents Coker 201 and *G. klotzschianum*, which were 1.5 kb and 900 bp and 900 bp and 600 bp, respectively. We used S1340 to detect the other ten hybrid plants which showed specific bands (1.5 kb) of Coker 201 plus the 600-bp band specific to *G. klotzschianum*. Banding patterns of the regenerants from the parental protoplasts were the same as those of the parents (Fig. 5b). It was therefore easy to distinguish plants regenerated from the parental protoplasts or from the somatic hybrids.

Discussion

The results obtained in this study show that protoplast fusion makes the production of somatic hybrids between *G. hirsutum* and *G. klotzschianum* feasible. To our knowledge, this is the first report of the production of such somatic hybrids through somatic hybridization. The genetic behavior of the progeny is under analysis.

There are 51 species of cotton in the world. Of these, 46 are diploid with the genomes of A, B, C, D, E, F, G and K and five are tetraploid with the genome of AD (Fryxell 1992). *Gossypium arboreum*, *G. herbaceum*, *G. hirsutum*, and *G. barbadense* are the four cultivated species and the others are wild species. Because cultivated cottons are highly susceptible to pests and diseases, they require intensive crop management. Although conventional breeding programs have made steady improvements in

Fig. 2a–i Plant regeneration from embryogenic callus, unfused protoplasts, and fused protoplasts via somatic embryogenesis. **a** Plant of Coker 201 regenerated from embryogenic callus. **b–g** Somatic hybrids. **h** Plant of Coker 201 regenerated from protoplasts. **i** Plant of *Gossypium klotzschianum* regenerated from somatic embryos

agronomic traits, not much genetic diversity in the cultivated tetraploids exists for further improvements (Kumria et al. 2003). There are, however, many valuable agronomic traits and abundant gene resources in the wild cottons. Unfortunately, the use of the wild species in breeding protocols is limited by poor compatibility and the sterility of interspecific hybrids. Biotechnological methods such as embryo rescue or interspecific cell fusion are required to overcome these barriers (Henn et al. 1998), but embryo rescue in cottons has resulted in plants produced at a low efficiency and with a low fertility, thereby limiting its use in breeding programs. Wild cottons have a great potential to widen the genetic diversity of cultivated cottons through somatic hybridization because this technology overcomes sexual crossing barriers. In this respect, somatic hybridization may be a viable alternative that will enable breeders to combine the genomes of incompatible species and to transfer nuclear or cytoplasmic traits such as CMS from one species to another (Atanassov et al. 1998; Cardi and Earle 1997).

Prerequisites for successful somatic hybridization include an efficient protoplast-culture scheme and a plant-regeneration scheme for at least one fusion parent (Krasnyanski et al. 1998). In preliminary studies, both Coker 201 and *G. klotzschianum* protoplasts were successfully isolated from different sources. Thus, unfused

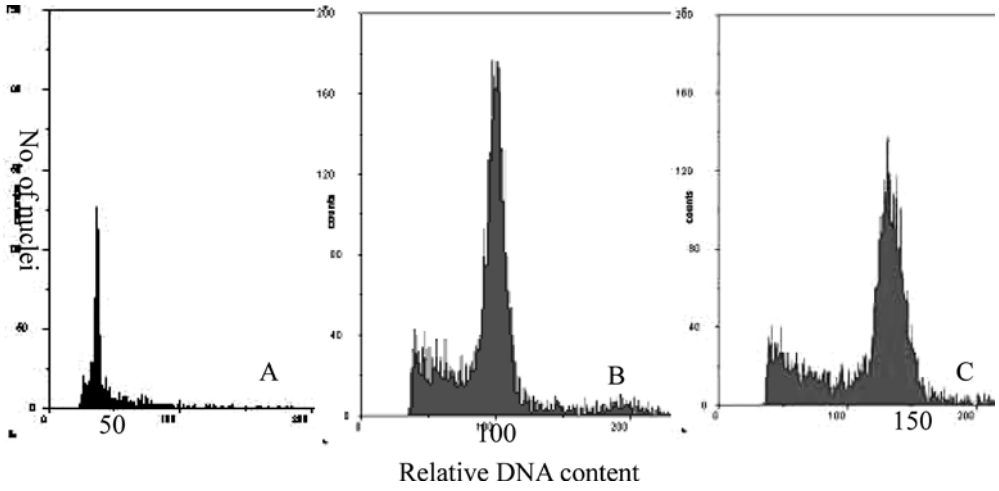

Fig. 3a–c Flow cytometry analysis of relative DNA content of leaf nuclei. **a** *G. klotzschianum* (parent). **b** *G. hirsutum* (parent). **c** Representative of somatic hybrid

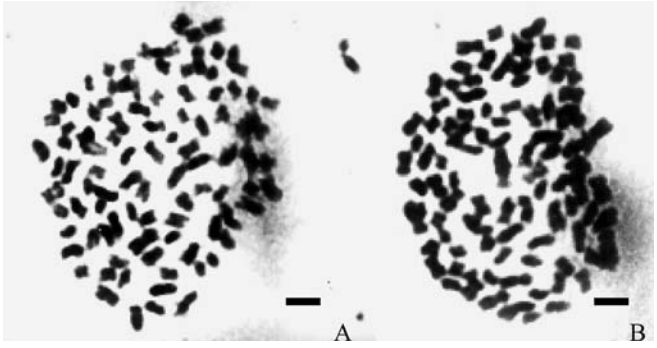

Fig. 4a, b Chromosome counts in root-tip metaphase cells. **a** Somatic hybrid of *G. hirsutum* (+) *G. klotzschianum*, 2*n*=78. **b** Aneuploid of *G. hirsutum* (+) *G. klotzschianum*, about 2*n*≈81. *Bar*: 50 μm

protoplasts with liquid thin-layer culture of the parents were used as a control, and regenerated plants were obtained. With a uniform cytoplasm membrane in the protoplasts, the fusion efficiency of the parents was very high (70%). The fusion efficiency varied from 22% to 70%, perhaps due to the origin and physiological condition of the parental protoplasts. Division efficiency, plating efficiency, and callus formation efficiency of the fused protoplasts were lower than those of the parents due to the effect of the electrical treatment.

Following fusion, the cultures yielded many microcalluses, which were initially placed in the dark and later under light. Many abnormal plantlets were produced from the 80 regenerated calluses, most of them having no normal growing points. Buds originated from dehiscent stems of many hybrids, and many roots formed from the stem apex, while the controls showed normal growth. Along with genetic distance and other factors, the unbalanced high ploidy level may play a major role in the variation of the hybrids. It is well known that chromosome reduplication is severely restricted by cell volume. In comparison to the controls and without any change in the growth conditions, polyploidization results in abnormal morphogenesis of the somatic hybrids.

Selection of the somatic hybrids was possible on the basis of morphological characters, cytological examination, and molecular analysis. Analysis of genomic DNA, genome size, and cytological behavior of the somatic hybrids showed that nearly a full genome of both parents was present in the recombined hybrid plants. Verification of somatic hybrid status was provided by flow cytometry. The relative DNA content of the hybrid plants was very close to the sum of the DNA contents of the parents. Thus, flow cytometry profiles from young leaves reflected the actual ploidy level of the plant. Repeated analyses provided similar results, thus showing a very high reliability of the technique. Chromosome counting also revealed that all of the tested somatic hybrid plants had a chromosome number ranging from 72 to 81, which was close to the expected 78 chromosomes [i.e., Coker 201 (2*n*=52) plus *G. klotzschianum* 2*n*=26)]. Almost 20 cells of each tested plant had a consensus.

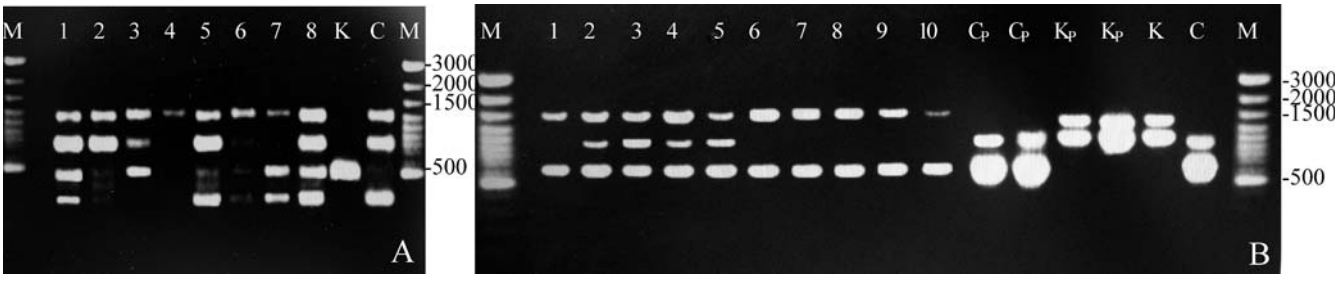

Fig. 5a, b RAPD patterns. **a** Primer S1325. **b** Primer S1340. Parental plants (*C G. hirsutum*, *K G. klotzschianum*, C_P regenerants from protoplasts of C, K_P regenerants from protoplasts of K) and putative somatic hybrids. *M* DNA marker, 3,000 bp

However, for plants in general, a deviation in the chromosome numbers in the somatic hybrids frequently involves polyploidization or the gain or loss of a few chromosomes, while, on the other hand, many sexual and somatic hybrids from distantly related plant species are unstable and tend to lose chromosomes from one genome (Miranda et al. 1997). At the same time, it was very difficult to be precise in counting the cotton chromosomes because of the high numbers involved.

Because somatic hybridization is a random genomic recombination process and the genome composition of the somatic hybrids is not well known, RAPD markers are a necessary and efficient molecular tool for estimating hybridity (Naoki et al. 1994; Xu et al. 1993). Well-defined RAPD markers are also useful for monitoring the stability of the offspring of somatic hybrid plants. In this study, seven of the ten RAPD primers used yielded products that were non-polymorphic. The genome of Coker 201 consists of the $(AADD)_1$ genome type, while the genome of *G. klotzschianum* consists of $(DD)_{3\text{-}k}$, and it is possible that D_1 and $D_{3\text{-}k}$ were highly homologous.

Following protoplast fusion of Coker 201 and *G. klotzschianum*, approximately 1,000 plants from 80 calluses were regenerated. Overall, about 50 of 1,000 (5%) putative plantlets developed into normal plants. Eighteen plants were selected for PCR-RAPD analysis to confirm the hybrid status. Chromosome counting and ploidy level revealed that all of the plants tested were polyploid, while RAPD analysis revealed that 16 of 18 plants had at least one parent-specific band of both parents. Thus, we can confirm that at least 16 plants were somatic hybrids.

In the study reported here, we produced somatic hybrids between a cultivated and a wild cotton. It was possible to successfully transfer genes (genomes) from wild species to breeding lines of cotton. This method can create novel cultivars, thereby providing plentiful materials for the study of genetics and breeding. Somatic hybridization can be applied to most species, but somatic hybrids frequently have a complex genetic constitution and many backcrosses may be required for the establishment of a new cultivar. In addition, these hybrids frequently suffer from chromosome instability and a high degree of sterility (Binsfeld et al. 2000). For successful application of these somatic hybrids for the improvement of cotton crops, backcrosses with the local cultivars will have to be performed to eliminate undesirable traits and sterility. The true test will be the stability of the hybrids over several generations. This stability may only be possible with $2n$=78 hybrids. The most powerful aspect of somatic hybridization is the aneuploid resource. The hybrids may also prove useful for the production of monosomic or disomic addition plants by microprotoplast fusion and recombinant lines after backcrossing (Ramulu et al. 1996).

Acknowledgements The author Y.Q. Sun thanks L.P. Ke, L.F. Zhu, and L.L. Tu for technical assistance with the molecular analysis and C.L. Cheng and J.K. Song for help on chromosome counting and flow cytometry. This research was supported by funds provided by the Chinese Ministry of Education.

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