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Somatic hybrids between *Gossypium hirsutum* L. (4×) and *G. davidsonii* Kellog (2×) produced by protoplast fusion

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Abstract Somatic hybrids were obtained from electrofused protoplasts derived from embryogenic suspension cultures of tetraploid cotton (*G. hirsutum* L. cv. Coker 201) and embryogenic callus of diploid wild cotton *G. davidsonii*. The regenerants were initially identified as hybrids by RAPD (random amplified polymorphic DNA) analysis. Subsequently, observation on chromosome counting, morphology and SSR (simple sequence repeat) confirmed the hybrid status. Cytological investigation of the metaphase root-tip cells of the regenerated plants revealed there were 74 to 84 chromosomes in the plants, close to the expected 78 chromosomes. SSR analysis revealed the regenerated plants contained specific genomic fragments from both fusion partners, further confirmed their hybridity. The morphology of the plants was intermediate between the two fusion partners. The regenerants were difficult to develop into mature plants because their roots browned and they wilted from the stem apex before forming 3 to 5 true leaves. The hybrid plants were transferred to soil by grafting *in vitro* onto rootstocks.

Keywords *Gossypium hirsutum* · *G. davidsonii* · Protoplast fusion · Somatic hybrid · RAPD · SSR

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Introduction

Mordern plant breeding practice has dramatically increased the productivity of major crops. Despite these achievements, genetic variation in breeding stocks still play an important role for further genetic improvement in cotton breeding. Chiefly a fiber crop, cotton contributed greatly to economic development both in China and the world. The cultivated cottons are highly susceptible to biotic and abiotic stresses in the long growth duration and require intensive crop management (Kumria et al., 2003). Although conventional breeding programs have made steady improvements in agronomic traits, it becomes more and more difficult to develop new varieties using current varieties because of poor genetic diversity in China (Wang et al., 2002). Most of the problems facing cotton production today are related to pests, diseases, and their controls (Wang et al., 2004, 2005). In light of the critical need to increase diversity in the gene pool, cotton improvement programs are increasingly turning to the application of molecular approaches to breeding and germplasm utilization (Mishra et al., 2003). One option to increase the genetic variability of cotton is to introduce genes of interest from its related wild species. Rich genetic sources are available in wild and wild relatives of cotton. Many wild cotton species have valuable agronomic traits such as disease and insect resistance and salinity and drought tolerance (Liang, 1999a). Genetic improvement through interspecific hybridization is hampered by incompatibility

barriers, and successful interspecific hybridization between wild species and cultivars has been limited by the necessity of embryo rescue. The low efficiency of embryo rescue and the low fertility of the resulting F_1 hybrids are both limiting factors for using hybridization techniques in certain breeding methods (Liang, 1999a). In such cases, if the genes of interest have been cloned, genetic transformation would be the method of choice; otherwise, symmetric/asymmetrical somatic hybridization through protoplast fusion offers an alternative approach for the introgression of the genes from wild species to commercial cultivars. Somatic hybridization is also potentially useful for the transfer of desirable traits – especially those controlled by polygenes or uncloned genes – from wild species to crop plants and for generating novel gene combinations by 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 & Earle, 1997).

Somatic hybridization by protoplast fusion has a great impact on crop breeding, such as in *Brassica* (Cardi & Earle, 1997), maize and wheat (Szarka et al., 2002), *Medicago* (Tian & Rose, 1999), and rice (Liu et al., 1999). We have recently reported the production of somatic hybrids between *G. hirsutum* L. and wild cotton (*G. klotzschianum*, *G. bickii* and *G. stockii*) (Sun et al., 2004; 2005). In this paper we report another somatic hybrid between *G. davidsonii* and *G. hirsutum* via protoplast fusion, with the aim to eventually transfer of multiple stress tolerance genes from *G. davidsonii* to *G. hirsutum*. Morphological and cytological analyses, as well as molecular markers were used to confirm the hybridity of the regenerated plants

Materials and methods

Plant materials

Embryogenic calli were derived from 5- to10 mm-long hypocotyl sections of *G. davidsonii* on MSB media [MS medium (Murashige & Skoog, 1962) supplemented with B_5 vitamin (Gamborg et al., 1968)] containing 0.9 μM 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 2.3 μM kinetin. They were subsequently transferred onto MSB medium containing 6.8 mM glutamine, 3.8 mM asparagine, 0.25% (w/v) Phytagel, and 3% (w/v) glucose, with 2.5 μM IBA (indole-3-butyric acid), and 0.46 μM kinetin for subculturing

The induction of embryogenic callus and establishment of suspension cultures of Coker 201 were described earlier (Sun et al., 2004). 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 provided by daylight fluorescent tubes at an irradiance of approximately 33 μmol m^{-2} sec^{-1})

Isolation and fusion of protoplasts

The protoplasts of Coker 201 were isolated from the suspension cultures, and the protoplasts of *G. davidsonii* were isolated from embryogenic callus. Procedures used in this experiment were described previously (Sun et al., 2004). The enzyme mixture was: 3% (w/v) Cellulase 'Onozuka' R-10 (Yakult Honsha, Tokyo, Japan), 1.5% (w/v) pectinase (Serva, Heidelberg, Germany), and 0.5% (w/v) hemicellulase (Sigma, California, USA). The protoplast viability was monitored by FDA (Fluorescein Diacetate) dyeing. The protoplasts were resuspended in the electrofusion solution containing 0.50 mM mannitol and 0.25 mM $CaCl_2$ to a density of 1×10^6 per ml. The protoplasts of Coker 201 and *G. davidsonii* were mixed at a 1:1.5 $\sim$ 2 ratios. An SSH-2 somatic hybridizer (Shimadzu Corporation, Toyota, Japan) was used to mediate fusion of the mixed protoplasts. About 1.6 ml of the mixed protoplasts was pipetted into the FTC-4 fusion chamber. The mixed protoplasts were induced to fuse in the electric field as the procedure described earlier (Sun et al., 2004). The protoplasts were then kept still for 10 to 15 min in order that the fusion products could regain normal shape, followed by centrifugation at 700 *g* for 6 min

Protoplast culture

The fusion-treated protoplasts were cultured in KM_8P medium (Kao & Michayluk, 1975) supplemented with 0.5 mM mannitol, 3% (w/v) glucose, 2.685 μM NAA (α-naphthaleneacetic acid), and 0.465 μM kinetin through a liquid thin layer culture with a final

density of 2.5×10^5 per ml. For the thin liquid layer culture, 4 ml of protoplast suspension was pipetted into 60 mm diameter plates, which were then sealed with parafilm and cultured in darkness at 28 °C. After 20 days, fresh liquid medium without mannitol was gradually added to the cultures to dilute by 50% concentration of mannitol to promote cell division. When calluses formed, they were transferred to solid MSB medium, which supplementing with 0.054 μM 2, 4-D, 0.233 μM kinetin, containing 3% (w/v) glucose and 0.25% (w/v) Phytagel (pH 5.8). Plant regeneration from callus was carried out on MSB culture medium with 0.555 mM myo-inositol, 0.27% (w/v) Phytagel, 3% (w/v) maltose, and 0.5% (w/v) activated charcoal, under light (14/10 h light/dark photoperiod with light provided by daylight fluorescent tubes at an irradiance of approximately 33 μmol m^{-2} sec^{-1}) at 30 °C. When the plantlets regenerated, the leaves were analyzed using a flow cytometer (Partec, Münster, Germany) as our report (Sun et al., 2004), which primarily confirmed the tested plants as hexaploid. The regenerated plants without strong roots were grafted onto the rootstock of *G. hirsutum* L. cv. Coker 201. Firstly, the rootstock plants were germinated from seeds and grown in pots until very well established roots and two true leaves had developed. Secondly, the in vitro regenerated plants were cleaved to cuniform, and the plant in the pot was cleaved at the same time. Then the cuniform was grafted onto the seed-developed rootstock with Parafilm binding and the grafted plants were grown in a greenhouse.

Chromosome preparation

For chromosome counting, 1-cm-long young root tips were collected from putative hybrids of non-grafted plants and the chromosome counting performed as previously described (Sun et al., 2004). Chromosomes were counted in about 20 metaphase cells of each line under a microscope.

Nuclear DNA analysis by RAPD and SSR

Regenerated plants were assessed for DNA polymorphism, using RAPD and SSR. Genomic DNA was extracted from young leaves following Bushra et al. (1999) and RAPD and SSR procedures followed Wu et al. (2003).

Results

Fused protoplast culture and plantlet regeneration

In the protoplast fusion mixtures, increasing the protoplast ratio of *G. davidsonii* and Coker 201 to $1.5 \sim 2{:}1$ promoted the frequency of hetero-fusion and homo-fusion of *G. davidsonii*. In the fused protoplast culture, the homo-fusions of *G. davidsonii* generally failed to form callus, and 30 individual calluses were formed from fused protoplasts in KM_8P medium. The light yellow callus was transferred to the solid media separately, embryogenic structures were produced in about 4 weeks, and then plantlets were produced via somatic embryogenesis. More than 20 normal plants from different callus-clones with 2 to 4 true leaves were obtained in 6 months. Using flow cytometry these were primarily determined to be hexaploids, as expected between the diploid (*G. davidsonii*, $2n = 26$) and tetraploid (*G. hirsutum*, $2n = 52$) parents. Eight of these were randomly selected for hybrid assessment.

Assessment of somatic hybrids

The eight tested plantlets were distinguished from the fusion partners through RAPD analysis using primers S461 (5′-GTAGCACTCC-3′) and S462 (5′-TCGGCACGCA-3′) (Sangon, Shanghai, China). When parental DNA was amplified by the two primers, the RAPD patterns were distinctly different (Fig. 1). The presence of distinct bands from both parents in the tested plantlets confirmed their hybrid status. These plants were further analyzed.

Young roots of six plants were collected from the eight hybrids for chromosome counting. Cells of 3 plants had just 78 chromosomes ($52 + 26$; Fig. 2A), as expected. Three hybrids showed aneuploidy with chromosome number varied from 74 to 84 (Fig. 2B-D). Variation of chromosome number including spontaneous chromosome elimination in somatic hybrids is a normal phenomenon, especially arising from distantly related species (Binsfeld et al., 2001; Miranda et al., 1997; Sun et al., 2004). It was very difficult to be precise in counting the cotton chromosomes because of the large numbers involved.

Additional verification for somatic hybrid status was provided by SSR analysis using three pairs of primers: BNL3280F (5′-GCAGAACTGCCACTT

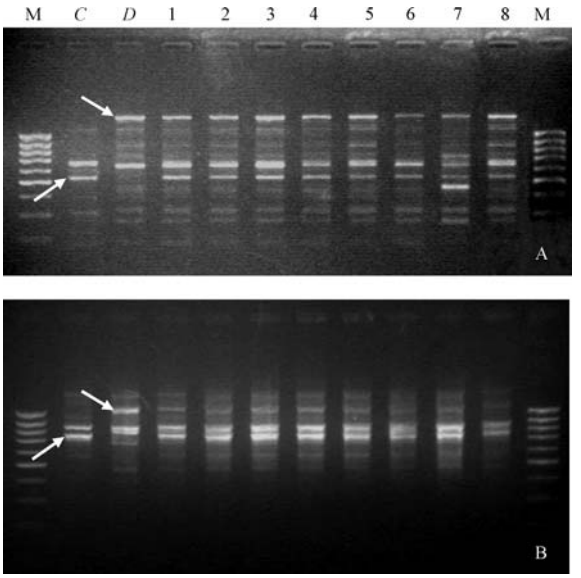

Fig. 1 A-B RAPD analysis of somatic hybrid plantlets. A-B band patterns of the parental species and regenerated plantlet by primers S461 (5′-GTAGCACTCC-3′) and S462 (5′-TCGGCACGCA-3′). Lane C = Coker 201, lane D = *G. davidsonii*, lanes 1–8 = eight independently derived regenerated plants, lane M = 1000 bp DNA ladder. Arrows indicate specific bands of fused parents

GTTTG-3′) and BNL3280R (5′-AGAAAATGGG-TTGTGCTTGG-3′); BNL2495F (5′-ACCGCCATTA-CTGGACAAAG-3′) and BNL2495R (5′-AATGGAA-TTTGAACCCATGC-3′); and BNL4059F (5′-GAG-TTACGCCTGGCAATCAT-3′) and BNL4059R (5′-CCATCCCCAGTGGTGTTATC-3′). The SSR patterns were distinctly different in the parents and the hybrids. The presence of distinct parental bands existed in the tested plantlets, with at least one typical band from each fusion partner present in the somatic hybrids tested (Fig. 3A,C), but one plantlet (No. 8) showed a band from parent Coker 201 only and nothing from *G. davidsonii* (Fig. 3B). The presence of banding patterns from both fusion partners in somatic hybrids confirmed that they contain DNA from both fusion partners. As each of the hybrids was from separate callus, the results implied the experiments were repeatable.

Morphological characteristics of somatic hybrids

After somatic embryos germinated, the roots were easily browned and when the plants had 2 to 4 true leaves, the stem apex became wilted and finally the whole plant wilted (Fig. 4). After the main stems were wilted, the new buds or callus appeared from the junction areas of plantlets and medium. In order to maintain plants, we grafted the hybrid shoots to a rootstock of Coker 201 (Fig. 4 F). The leaves of regenerated plants were entire, heart or elliptic shape. They had broad and thick leaves, which are typical of somatic hybrids and polyploids, with morphology intermediate between those of fusion partners (Fig. 5). The grafted plants kept alive and rooted well within 4 weeks (Fig. 6) indicating a potential way to obtain surviving hybrids. Over 10 somatic hybrid plants were independently derived from different callus origins were transferred to soil (Fig. 6).

Discussion

There are 51 species in *Gossypium*, among them 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). Although conventional breeding programs have made steady improvements in agronomic traits in cultivated tetraploids, further improvement is limited because of low levels of genetic diversity (Kumria et al., 2003). There are many valuable agronomic traits and abundant gene resources in the wild cottons. However, the use of wild species in breeding programs is limited by poor compatibility and sterility of interspecific hybrids. Biotechnological methods such as embryo rescue or interspecific cell fusion are required to overcome these barriers (Henn et al., 1998), 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 & Earle, 1997).

In this study, the fused cultures yielded about 30 clones of callus. Over twenty plantlets were produced from the regenerated calluses *via* somatic embryogenesis, many abnormal plantlets produced. The morphology of regenerated plants was intermediate between the fusion partners. This study and the previous report (Sun et al., 2004, 2005) indicated it is

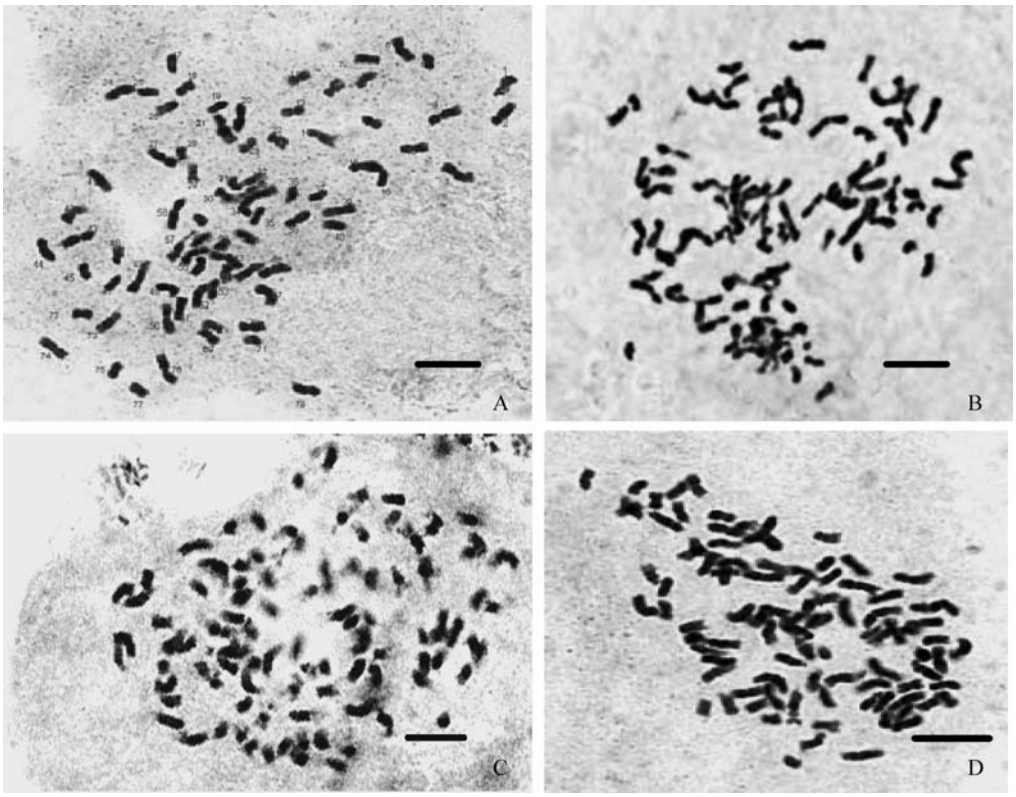

Fig. 2 A-D Chromosome counts in root-tips metaphase cells. A Somatic hybrid of *G. hirsutum* (+) *G. davidsonii*, 2n = 78, B Aneuploid of *G. hirsutum* (+) *G. davidsonii*, 2 n ≈ 82, C Aneuploid of *G. hirsutum* (+) *G. davidsonii*, 2 n ≈ 76, D Aneuploid of *G. hirsutum* (+) *G. davidsonii*, 2 *n* ≈ 84 (bar = 50 μm)

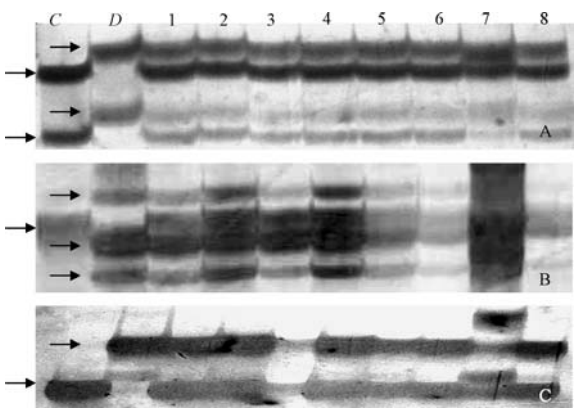

Fig. 3 A-C SSR analysis of fusion parents and somatic hybrid plantlets. A-C band patterns of the parental species and regenerated plantlet by the pairs of primers, BNL3280.F (GCAGAACTGCCACTTGTTTG)/BNL3280.R (AGAAAATGGGTTGTGCTTGG), BNL2495.F (ACCGCCATTACTGGACAAAG)/BNL2495.R (AATGGAATTTGAACCCATGC) and BNL4059.F (GAGTTACGCCTGGCAATCAT)/BNL4059.R (CCATCCCCAGTGGTGTTATC). Lane *C* = Coker 201, lane *D* = *G. davidsonii*, lanes 1–8 = eight independently derived regenerated plants. Arrows indicate specific bands of fused parents

feasible to obtain somatic hybrids between cultivated and wild species of cottons via protoplast fusion

Selection of somatic hybrids was possible according to morphological characters, cytological examination, and molecular biology. The regenerants were firstly detected as polyploids by flow cytometry and somatic hybrids by RAPD. Further analysis of genomic DNA with SSR markers and cytological behavior of the somatic hybrids showed that nearly a full genome of both parents was present in the recombined hybrid plants. But in SSR analysis (Fig. 3B), banding pattern of one plantlet (No. 8) showed only from one parent Coker 201. Chromosome counting also revealed that all the tested somatic hybrid plants had a chromosome number ranging from 74 to 84, close to the expected 78 chromosomes (i.e., Coker 201 $2n = 52$ plus *G. davidsonii* 2n = 26). This may either represent aneuploidy or difficulties in accurately counting the small chromosomes.

Production of interspecific hybrids by protoplast fusion would be an effective way to creating novel cultivars, providing plentiful materials for the study

Fig. 4 Regenerated somatic hybrid plants. A Plantlets with browning roots. B-D Process of wilting of regenerated hybrid plantlets. E Plants through grafting *in vitro* rooting well. F. The plant transferred to the soil

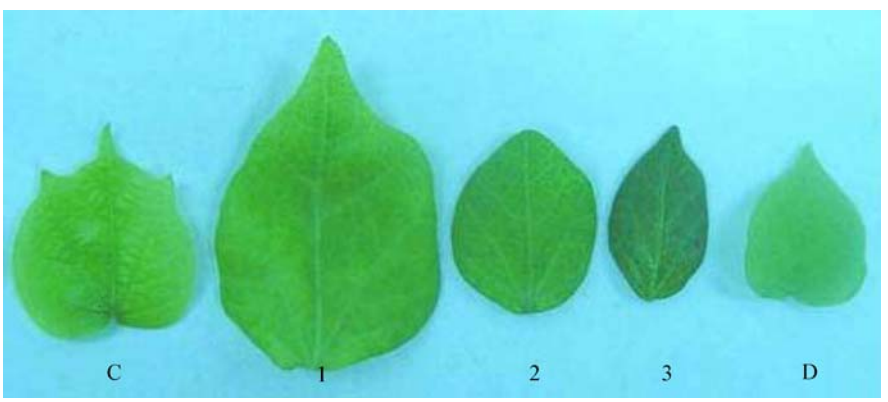

Fig. 5 Leaf morphology of parental species and somatic hybrid plants. C = leaf of Coker 201; 1, 2 and 3 represent leaves of somatic hybrid plants; and D = leaf of *G. davidsonii*

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 somatic hybrids for improvement of cotton crops, backcrosses with the local cultivars will have to be performed to eliminate undesirable traits and sterility. The hybrids may also prove useful for the production of monosomic or disomic addition lines by microprotoplast fusion and recombinant lines after backcrossing (Ramulu et al., 1996).

We have obtained somatic hybrids between *G. hirsutum* and *G. klotzschianum*, *G. bickii* and *G. stockii* (Sun et al., 2004, 2005), and we intended to broaden somatic hybrids between *G. hirsutum* and other wild species in this study. Among these combinations, somatic hybrids between *G. hirsutum* and *G. davidsonii* became wilted when they developed to 3 to 5 true leaves. In the sexual hybridization of *G. arboreum* L. and *G. davidsonii*, many researchers found the hybrid embryos almost did not develop normally, and formed

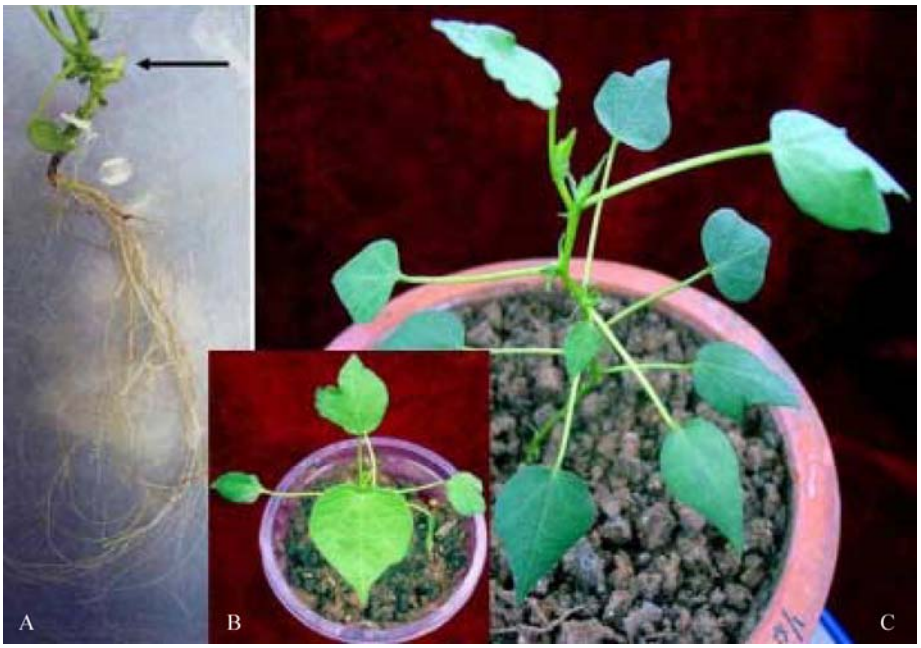

Fig. 6 Somatic hybrid plants transferred to the soil. A The very well roots of somatic hybrid plant (the arrow indicates the position of the graft), B-C Somatic hybrid plants transferred to the soil

various abnormal embryos (He & Liang, 1989). He and Liang (1989) concluded that the death of hybrid embryos between *G. arboreum* L. and *G. davidsonii* took place at the globular-shaped stage or cotyledon differentiation stage. In the sexual hybridization of four cultivars and *G. davidsonii*, the phenomena of cotyledon putrescence and embryo death existed regardless of which of the four cultivars as female parent; the plants in cuvettes obtained by embryo rescue could not be successfully transferred to field. The previous researches indicated that putrescence of embryos began with mitochondria putrescence in the ultrastructure level, followed by cell putrescence, then the whole embryo putrescence (Liang, 1999b). Therefore, it is clearly very difficult to obtain sexual hybrids of *G. davidsonii* and cotton cultivars through distant hybridization and embryo rescue. The somatic hybrids of *G. hirsutum* and *G. davidsonii* obtained through protoplast fusion in our study, allowed the problem of embryo putrescene to be circumvented. Although the young leaves wilted at 3–5 leaves stage and the whole plants then languished, we successfully obtained hybrid plants with more than 10 leaves by grafting hybrid shoots to rootstocks of Upland cotton. This is the first report of hybrids between *G. hirsutum* and *G. davidsonii* and will therefore contributes to cotton breeding and genetic research. The wild cotton *G. davidsonii* has the characteristics of alkali- and salt-tolerance, resistance to bacterial diseases and aphids. These traits are very useful for genetic improvement of cotton cultivars.

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