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Characteristics of fertile somatic hybrids of *G. hirsutum* L. and *G. trilobum* generated via protoplast fusion

X. S. Yu · B. J. Chu · R. E. Liu · J. Sun ·
Joseph Jones Brian · H. Z. Wang · Zhu Shuijin ·
Y. Q. Sun

Abstract Fertile somatic hybrids between tetraploid upland cotton *G. hirsutum* L. cv. Coker 312 and wild cotton *G. trilobum* were generated by symmetric electrofusion. Comparisons of morphology, combined with flow cytometric, RAPD, SRAP and AFLP analyses confirmed the hybrid nature of the regenerated plants. The hybrids differed morphologically from the parent plants. Flow cytometric analysis showed that the hybrids had DNA similar in amount to the total combined DNA content of the two parents, and the use of molecular markers revealed that the hybrids contained genomic fragments from both fusion parents, further indicating the hybrid nature of the regenerated plants. The stability of the morphological features of the hybrids was examined in following generations. The hexaploid fusion plants showed strong photosynthesis and a high expression level of some photosystem-related genes.

Our results suggest that novel traits may be incorporated in cotton breeding programs through the production of somatic hybrids and the backcrossing of these plants with elite cultivars.

Abbreviations

MS medium	Murashige and Skoog medium (1962)
NAA	Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
KT	Kinetin
RAPD	Random amplified polymorphic DNA
SSR	Simple sequence repeats
AFLP	Amplified fragment length polymorphism
SRAP	Sequence related amplified polymorphism

X. S. Yu, B. J. Chu and R. E. Liu contributed equally to this work.

X. S. Yu · B. J. Chu · R. E. Liu · H. Z. Wang · Y. Q. Sun (✉)
College of Life and Environmental Science, Hangzhou
Normal University, Hangzhou 360016, Zhejiang, China
e-mail: yqsun@hznu.edu.cn

J. Sun
College of Agronomy, Shihezi University,
Shihezi 832000, Xinjiang, China

J. J. Brian
Faculty of Agriculture, Food and Natural Resources,
The University of Sydney, A20 John Woolley Building,
Sydney, NSW 2006, Australia

Z. Shuijin
College of Agriculture and Biotechnology, Zhejiang University,
Hangzhou 310029, Zhejiang, China

Introduction

Tetraploid upland cotton (*Gossypium hirsutum* L.) is a globally important crop grown primarily for its fiber, and its production is a vital part of the economy, politics, and society in many countries. Cotton is highly susceptible to a wide variety of biotic and abiotic stresses. It is becoming increasingly difficult for breeders to develop new varieties to meet emerging challenges because of the limited diversity in the germplasm of the commercially cultivated, upland cotton. A rich reservoir of genetic material, much of it with potentially valuable agronomic traits, is available in wild cotton species. Traits from wild cotton species such as drought tolerance and resistance to bacterial diseases could provide valuable additions to breeding programs (Liang 1999a). What is needed is an efficient means of incorporating this genetic material into upland cotton.

Conventional breeding methods (Henn et al. 1998; Liang 1999b) continue to result in the incorporation of new traits, however, it would be beneficial if additional methods were used to extend, facilitate and expedite the incorporation of genetic material from evolutionarily distant species into upland cotton breeding programs. Somatic hybridization by protoplast fusion has had considerable impact on the breeding of a variety of crop species as it facilitates the transfer of genetic information from disparate, sexually incompatible species. We have previously published work showing the potential for somatic hybridization between wild cottons containing important traits to elite upland cotton cultivars and have obtained novel combinations of nuclei and cytoplasmic organelles (Sun et al. 2004, 2005, 2011; Sun and Zhang 2006; Yang et al. 2007). We have also shown that somatic protoplast hybrids between tetraploid cotton (Gossypium hirsutum L. cv. Coker 201) and diploid wild cottons (G. klotzschianum, G. bickii and G. stockii) can be successfully regenerated into viable plants containing novel combinations of genetic material.

In this paper, we report the successful fusion of protoplasts derived from G. hirsutum L. cv. Coker 312 and the wild cotton species, G. trilobum (Mocono & Sesse ex DC) Skovsted. G. trilobum is diploid with the genome D_8 ($2n = 2x = 26$). The seeds were kindly supplied by Dr. Kunbo Wang (Cotton Research Institute of Chinese Academy of Agricultural Science). G. trilobum has been shown to have many valuable traits, including a tolerance to drought, cotton bollworm, pink worm, and boll rot, and a strong resistance to Verticillium and Fusarium wilt. It is also particularly tolerant to short-period, low temperature (frost) damage. Upland cotton lacks these potentially valuable traits. The aim of this work was to obtain somatic hybrid plants in order to incorporate the useful traits from G. trilobum into upland cotton breeding programs. The somatic hybrid plants obtained were analyzed by their ploidy, morphological characteristics, and molecular composition in order to confirm their hybrid status. We also show that, interestingly, the somatic hybrids between G. hirsutum and G. trilobum have a high photosynthetic capacity and strong vegetative growth.

Materials and methods

Plant materials

Embryogenic calli of G. hirsutum L. cv Coker 312 were obtained from 1 to 2 cm long hypocotyls of 7-day sterile seedlings on a modified MSB medium (MS inorganic salts and B_5 vitamins) supplemented with 3 % (w/v) glucose, 0.25 % (w/v) Phytagel, 0.01 mg/L 2,4-D, 0.15 mg/L KT.

The calli were subsequently subcultured in modified MSB medium (KNO_3 were doubled) with added 3 % (w/v) glucose, 0.25 % (w/v) Phytagel, 0.5 mg/L IBA, 0.15 mg/L KT. Embryogenic calli of G. trilobum were produced after 6–7 subcultures (about 5 months) on MSB medium supplemented with 3 % (w/v) glucose, 0.25 % (w/v) Phytagel, 0.01 mg/L 2,4-D, 0.5 mg/L IBA, 0.15 mg/L KT. Subsequently somatic embryos of G. trilobum were formed on MS medium containing 0.5 mg/L IBA and 0.15 mg/L KT and used for protoplasts isolation. For Coker 312, embryogenic calli were inoculated into 40 mL modified liquid MSB medium containing 0.1 g/L glutamine, 0.1 g/L asparagines, 0.1 g/L KCl, 0.1 g/L NaCl and 3 % (w/v) glucose to establish suspension cultures for protoplast isolation.

Isolation and fusion of protoplasts

The protoplasts of Coker 312 and G. trilobum were isolated from suspension cultures and somatic embryos. Suspension cultures were in the first instance digested in filter-sterilized enzyme solutions [3 % (w/v) Cellulase R-10, 1 % (w/v) macerozyme dissolved CPW9 solution with 9 % (w/v) mannitol] at 28 °C in the dark for 20 h. To ascertain protoplast viability, we stained the protoplasts with fluorescein diacetate (FDA) after purification, as previously described (Sun et al. 2004). Purified protoplasts of the two species were mixed at a ratio of 1:1.5 (Coker 312 and G. tribolum) to give a total concentration of 5×10^5 protoplasts/mL for electrofusion. Protoplasts were fused using a BTX Inc square-wave pulse generator as described in Sun et al. (2011). After the electrofusion treatment protoplasts were kept undisturbed for 30 min to regain their normal morphology, followed by centrifugation at $50 \times g$ for 5 min.

Protoplast culture and plant regeneration

Centrifuged protoplasts were resuspended in 1.3 mL of filter-sterilized modified KM8P medium (Kao and Michayluk 1975) containing 2,4-D 0.01 mg/L, 0.1 mg/L NAA and KT 0.15 mg/L adjusted to a density of approximately 2×10^5 cells/mL. During the first month of culture, the petri dishes were incubated in darkness at 30 °C and fresh liquid KM8P medium with 4.5 % (w/v) of mannitol was added after 2 weeks to reduce osmolality. Protoplasts cultures were stored at 30 °C in complete darkness. Callus induction was carried out under light conditions (14/10 h-light/dark photoperiod) with light provided by daylight fluorescent tubes at an irradiance of approximately 33 umol m^{-2} s^{-1} at 28 °C.

When microcalli formed, they were proliferated in modified MSB medium plus 0.01 mg/L 2,4-D, 0.15 mg/L KT and then transferred onto embryogenic callus-induced

medium (MS media with B5 vitamins and KNO$_3$ doubled, supplemented with 0.5 mg/L IBA, 0.15 mg/L KT) until regenerated plantlets were formed. The plantlets were cultured continuously for approximately 4–6 weeks on basal MS medium. When the plants had 3–5 true leaves with many strong roots, they were transferred to a potting mix soil (soil:sand 3:1) and placed in the greenhouse with a relative humidity of 75 % and a light period of 12 h. If the roots of the plantlets were too weak, the plantlets were grafted onto a Coker 312 rootstock in the greenhouse as previously reported (Sun and Zhang 2006). Hybrid plants were subsequently artificial supplementary pollinated 2–3 times and GA$_3$ (25 µg/mL) was spread on the petioles of the bolls in order to get bolls and seeds.

Primary selection of callus formed from the fused protoplasts

Approximately 0.2 g of callus from each of the lines of interest, Coker 312, *G. trilobum*, and newly formed calli from the fused protoplasts were ground separately and analyzed for DNA content using a flow cytometer (Partec, Münster, Germany). Regenerated callus with a relative DNA content close to the sum of the DNA content of Coker 312 and *G. trilobum* was preliminarily regarded as somatic hybrid callus and was subcultured.

Somatic hybrid selection

Ploidy was further analyzed after plantlets formed. Approximately 0.5 g of young leaves from Coker 312 and *G. trilobum*, and the putative hybrid regenerants were chopped and analyzed using a flow cytometer (GmbH D-48161, Partec, Munster, Germany) according to the procedure described in Sun et al. (2004). Each histogram was generated by the analysis of at least 10,000 cells and repeated three times. Flow cytometer analysis of material from the putative hybrid plantlets further indicated that they were *G. hirsutum* + *G. trilobum* hybrids. Plantlets from the same clone were analyzed further by cytological and genomic DNA analysis.

Somatic hybrid genomic analysis using RAPD, SRAP, SSR and AFLP

Total genomic DNA was extracted from the two parental lines and the somatic hybrid lines according to the method described in Paterson et al. (1993). RAPD, SRAP and AFLP analyses for hybridity were carried out as described in Sun et al. (2004), Lin et al. (2005), and Hawkins et al. (2005). GeneRuler 100-bp DNA Ladder Plus (Sangon, Shanghai) was used as the DNA marker. From the RAPD random primers, the amplification patterns of the two

primers of S174 and S197 were 5′-AGTGCACACC′ and 5′-ACACTCGGCA-3′, which showed the parental bands from both parents present in the somatic hybrids. The SSR profiles were generated using primers for NAU1092 (forward 5′-ACTCAAGCTTGGTTGGAAAG-3′, reverse 5′-CCTTGAGGCATTAGGTATCG-3′) and NAU1256 (forward 5′-TGGCTTTCAATCATCAACAC-3′, reverse 5′-CAGTTGATTCCGTTCTTCCT-3′). Polymorphic bands were generated in the putative hybrid lines. The SRAP analysis was conducted using the primer combinations of M + EM (Table 1). The three combinations gave clearly different patterns as M5 + EM5, M8 + EM10 and M7 + EM7.

The AFLP protocol used was modified from the report of Hawkins et al. (2005). Approximately 200 ng of total genomic DNA was digested with *EcoR* I and *Mse* I in a 20 µL reaction. Pre-selective AFLP amplification was performed using a single selective nucleotide (+1) at the 3′ end of both the *Mse* and *Eco* primers (Table 2). Selective AFLP amplifications were performed by adding three selective nucleotides (+3) to the 3′ end of the *Mse* and *Eco* primers. Six +3 selective AFLP amplifications were performed using 3 primer combinations. The primers used were MC-AA, EA-TG, EA-GT and MC-AC, EA-TG, EA-GT. Amplification products were electrophoretically separated using a Sequi-Gen GT Nucleic Acid Electrophoresis cell (Bio-Rad), and AFLP images were analyzed by visual inspection. The AFLP PCRs were repeated two times.

Somatic hybrid mitochondrial and chloroplast analysis

To identify the pattern of mitochondrial and chloroplast inheritance, genomic DNA was extracted from the two parental lines and the somatic hybrid lines and analyzed using universal primers for mitochondrial and chloroplast DNA (Table 3). The polymerase chain reaction was carried out under the conditions: 94 °C 4 min, 94 °C 30 s, 60 °C 1 min, 72 °C 1 min, 35 cycles. Universal primer sequences are listed in Table 3.

Stomatal observation and chlorophyll content measurement

The youngest fully expanded leaf was cut from each parent and somatic hybrid plants. An abaxial epidermal peel was carried out with each leaf. The peeled epidermis was subsequently photographed under a phase-contrast stereomicroscope (Olympus BX51). The number of stomata and pavement cells were counted and a stomatal density was calculated for each leaf.

To assay Chl content, six leaf discs (approximately 0.4 g fresh mass) were cut from the youngest fully expanded leaf

Table 1 Primer for SRAP PCR

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
m1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
m2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
m3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
m4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
m5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTAAC
m6	TGAGTCCAAACCGGTAA	em6	GACTGCGTACGAATTGCA
m7	TGAGTCCAAACCGGTCC	em7	GACTGCGTACGAATTCAA
m8	TGAGTCCAAACCGGTGC	em8	GACTGCGTACGAATTCTG
		em9	GACTGCGTACGAATTCGA
		em10	GACTGCGTACGAATTCAG
		em11	GACTGCGTACGAATTCCA

Table 2 Primers for AFLP PCR

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
Eco RI F adapter	CTC GTA TAC TGC GTA CC	EA-AA	TAC TGC GTA CCA ATT C AAA
Eco RI R adapter	AAT TGG TAC GCA GTA	EA-AT	TAC TGC GTA CCA ATT C AAT
Mse I F adapter	GAC GAT GAG TCC TGA G	EA-AC	TAC TGC GTA CCA ATT C AAC
Mse I R adapter	TAC TCA GGA CTC ATC	EA-AG	TAC TGC GTA CCA ATT C AAG
Eco RI + 1 primer	TAC TGC GTA CCA ATT C A	EA-TA	TAC TGC GTA CCA ATT C ATA
Mse I + 1 primer	GAC GAT GAG TCC TGA GTA AC	EA-TT	TAC TGC GTA CCA ATT C ATT
Mse I + 3 primer_1	GAC GAT GAG TCC TGA GTA A CAA	EA-TC	TAC TGC GTA CCA ATT C ATC
Mse I + 3 primer_2	GAC GAT GAG TCC TGA GTA A CAC	EA-TG	TAC TGC GTA CCA ATT C ATG
Eco RI + 3 primer_1	TAC TGC GTA CCA ATT C AGC	EA-CA	TAC TGC GTA CCA ATT C ACA
Eco RI + 3 primer_2	TAC TGC GTA CCA ATT C ACG	EA-CT	TAC TGC GTA CCA ATT C ACT
Eco RI + 3 primer_3	TAC TGC GTA CCA ATT C AAC	EA-CC	TAC TGC GTA CCA ATT C ACC
Eco RI + 3 primer_4	TAC TGC GTA CCA ATT C ACA	EA-CG	TAC TGC GTA CCA ATT C ACG
		EA-GA	TAC TGC GTA CCA ATT C AGA

Table 3 Universal primers for mitochondrial and chloroplast amplification

	Primer1	Primer2
Chloroplast primer	*Rbc*L 5′-ATGTCACCACAAACAGAAACTAAAGCAAGT-3′	*Rbc*L5′-CTTCACAAGCAGCAGCTAGTTCAGGACTCC-3′
	*PSA*I 5′-TTTGGTGGAGGAACTTTAGGACACCCTTGGGG-3′	*PSA*I 5′-GCAATTGCCGGAAATACTAAGC-3′
	*Trn*H 5′-ACGGGAATTGAACCCGCGCA-3′	*Trn*K 5′-CCGACTAGTTCCGGGGTTCGA-3′
	*Trn*D 5′-ACCAATTGAACTACAATCCC-3′	*Trn*T 5′-CTACCACTGAGTTAAAAGGG-3′
	*Trn*K 5′-AACCCGGAACTAGTCGGATG-3′	*Trn*K 5′-TCAATGGTAGAGTACTCGGC-3′
Mitochondrial primer	*nad*1 exon B 5′-GCATTACGATCTGCAGCTCA-3′	*nad*1 exon C 5′-GGAGCTCGATTAGTTTCTGC-3′
	18S rRNA 5′-GTGTTGCT-GAGACATGCGCC-3′	5S rRNA 5′-ATATGGCGCAAGACGATTCC-3′
	*nad*4 exon 1 5′-CAGTGGGTTGGTCTGGTATG-3′	*nad*4 exon 2 5′-TCATATGGGCTACTGAGGAG-3′
	NTCP9 5′-CTTCCAAGCTAACGATGC-3′	NTCP9 5′-CTGTCCTATCCATTAAGACAATG-3′

of four replicate plants avoiding mid-rib vascular tissue. The cuts were uniformly distributed across the leaf. Discs were placed in 80 % acetone until all colour was extracted (approx. 3 h). Chl content of the solution was then assayed spectrophotometrically. Chl content is presented as per unit fresh leaf mass.

Measuring photosynthesis of somatic hybrids

Leaf photosynthetic characteristics were compared between the two parents and the somatic hybrid plants. Photosynthetic measurements were carried out on greenhouse-grown plants at the pre-flowering stage. Plants were grown under natural light (about 14 h day/10 h night). All measurements were made on the youngest fully expanded main stem leaf of pre-flowering plants. Measurements were repeated three times on four plants for each genotype at the following times: 9 a.m. (dawn + 4 h), 12 a.m. (dawn + 7 h), 15 p.m. (dawn + 10 h) and 18 p.m. (dawn + 13 h) (Lu et al. 1994).

The photosynthesis rate (μmol CO_2 m^{-2} s^{-1}) and transpiration rate (μmol m^{-2} s^{-1}) measurements were performed with a portable photosynthesis measuring system (Lu and Zeiger 1994). Stomatal conductance (μmol H_2O m^{-2} s^{-1}) was measured with a steady-state porometer (LiCor Instruments, Lincoln, NE; model Li-6400).

The data were analyzed using one-way ANOVA in combination with a Dunnett's comparison test.

Gene expression analysis for photosynthesis

RNA isolation

Total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN, USA). First-strand cDNA was synthesized from 20 μg of total RNA with an oligo (dT) 24 primer and Super Script II reverse transcriptase (Quanta). Transcripts were quantified by real-time RT-PCR analysis using 1/100th of the resulting cDNA as template. Real-time RT-PCR was performed with the StepOnePlus Real-time PCR system (ABI, USA) with SYBR Premix Ex Taq (Quanta).

Transcript levels for photosynthesis were assessed by three independent biological replicates in real-time PCR in triplicate with reaction mixtures (final volume, 20 μL) containing 5 μL of cDNA, 0.5 μM of both forward and reverse primers, and 1$\times$ FastStart SYBR Green Master mix (Roche). A balanced randomized block design was used for the real-time RT-PCR experiments (Rieu and Powers 2009). The following standard protocol was applied for the amplification of each mRNA: 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. The reference gene was S1F Sense CCAAAGGAGGTGCCTGTTCA, S2R Antisense TTGAGGTGAGTCAGAATGTTGTTC described by Yang et al. 2005. The six photosynthesis-related genes monitored were: *psaA* (photosystem I P700 chlorophyll a apoprotein A1), *psaB* (photosystem I P700 chlorophyll a apoprotein A2), *psbA* (photosystem II protein D1), *ccsA* (cytochrome c biogenesis protein), *ycf3* (photosystem I assembly protein ycf3), *ndhF* (NADH dehydrogenase subunit 5) with primer sequence as Table 4.

Expression in the wild type was calculated using the formula $E_R^{CT}{}_{WT}/E_T^{CT}{}_{WT}$ [i.e., $(1/E_T^{CT}{}_{WT})/(1/E_R^{CT}{}_{WT})$: the normalized relative quantity of template in the original sample], the expression levels of target genes being determined relative to those of the reference gene.

All real-time RT-PCR results are means and corresponding standard errors obtained for the three biological replicates and used as one technical replicate in the one-way ANOVA combined with a Dunnett's comparison test.

Analysis of gene expression by differential display

For the DD, cDNA was synthesized from the total RNA of Coker 312, *G. trilobum* and their somatic hybrids. cDNA–AFLP was used to analyze the differential expression in the two parental lines and derived the somatic hybrids.

The cDNA–AFLP protocol used was a modification of the original technique (Bachem et al. 1996), which permits the visualization of one single cDNA fragment for each messenger originally present in the sample, thus reducing redundancy of sequences obtained. The cDNA was digested and pre-amplified according to the procedure of Hawkins et al. (2005). AFLP analysis was carried out as described previously in this paper.

Results

Protoplast isolation, fusion, and regeneration

The protoplasts of *G. hirsutum* L. cv. Coker 312 and *G. trilobum* were hybridized by electrofusion using an Electroporator Electro Cell Manipulator (BTX ECM 2001). The heterofusion rate of approximately 10–15 % was lower than the value (approximately 30 %) previously obtained for SSH-2 in 2004 (Sun et al. 2004). After 3 months in KM8P medium, callus-clones were formed from the fused protoplasts (Fig. 1a), and each was transferred to a solid medium and numbered. When the microcalli developed to approximately 2 mm in diameter (Fig. 1b) it was transferred to the callus proliferation medium (Fig. 1c–d). A total of 34 putative fusion clones was obtained. Seven of these were shown to have a DNA content close to the sum of the two parents. These seven clones were numbered and selected for further subculturing. After 3–5 subcultures (at 3 weeks/subculture), some cotyledonary embryos with elongated radicles germinated and developed into plantlets and subsequently developed into plants with an overall normal gross morphology (Fig. 1e).

Morphological characteristics of the regenerated plants

Seven plants derived from the presumptive hybrid calli (34 clones) were successfully transferred to the soil though

Table 4 Primer of photosynthesis gene in cotton for real time PCR

Primer	Sequence (5′–3′)	Primer	Sequence (5′–3′)
ndhF-F	CCGGCGCAGTTGCTAAAT	ndhF-R	CCCTCCATGGCATCAGGTAA
ccsA-F	TTTGCCACTTCGGGTCTTTT	ccsA-R	TGGAGAGCAGGTGCTAATATTGC
psbA-F :	GGTTTGCACTTTTACCCGATCT	psbA-R	GGACCGCCATTGTATAACCATT
ycf3-F	AATCGAATGACGACCGTAACG	ycf3-R	AGGCCTCCGCATAATTTCCT
psaA-F	CATTGGTCTTTGCGGCCTTA	psaA-R	GGCCGCTTTGTGATAATGGA
psaB-F	CAACGAATGGCCCAGCAT	psaB-R	AACCAGCCAGGCAACCATAT
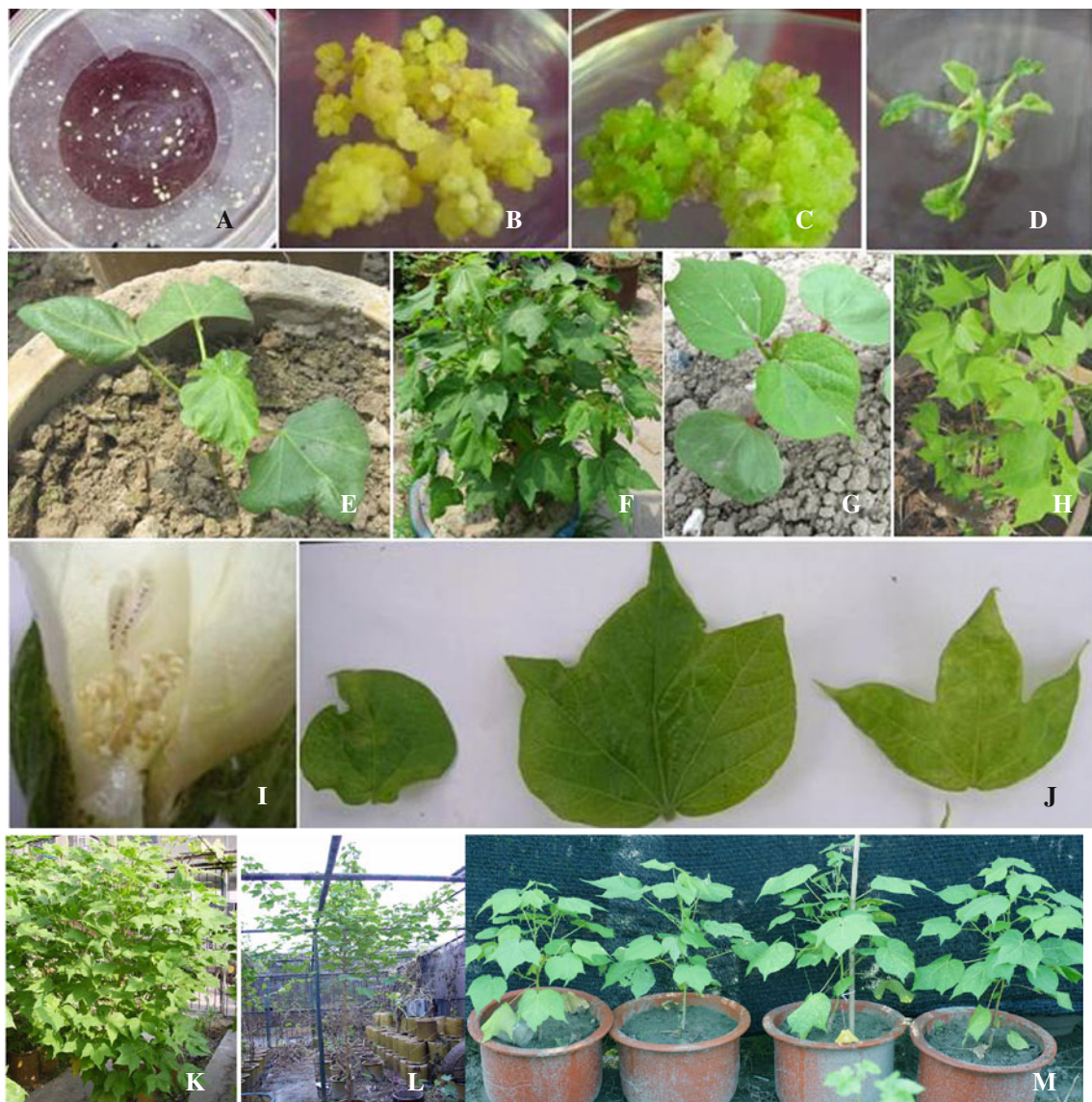
Fig. 1 Plants regenerated from fused protoplasts (Coker 312 + *G. trilobum*). **a** Proto-calli formed from protoplast liquid culture, **b** embryogenic calli proliferated from proto-calli on solid medium, **c** somatic embryo induction, **d–f** somatic hybrid plant regeneration, **g** Coker 312, **h** *G. trilobum*, **i** abnormal anther, **j** leaf morphology of Coker 312, **k,l** somatic hybrid growing in greenhouse, **m** the progeny of somatic hybrids regenerated plant and *G. trilobum*, respectively

direct transplant and in vitro graft and grown to maturity. Although normal, the morphology of somatic hybrid plants differed from that of the two parental plants (Fig. 1f–h). The hybrid plants were taller, and had a thicker stem and leaves that were thicker and deeper green than either of the wild-type parents. The shape of leaves also differed from the parent lines (Fig. 1j). After 2 months in the greenhouse, the somatic hybrids have many branches with many leaves (Fig. 1k), after 3 months, the somatic hybrid plants reached 2 m in height (Fig. 1l) with spreading branches. They produced many flower buds, which had abnormal calyxes, petals, and stigma (Fig. 1i). The hybrid plants flowered, but the stamens were withered and pollen viability was very low (~ 8 %). After cross-pollination with their pollen and application of GA$_3$ (25 µg/mL) on the petioles of the bolls, few seeds were obtained in these lines (Fig. 2b), covering with light white fiber and light yellow fuzz, different with the parental line Coker 312 (Fig. 2a) and wild cotton *G. trilobum* (Fig. 2c). The progeny of these hybrids grew with similar phenotypes to the previous generation (Fig. 1m).

Ploidy level

Leaves were collected from plants derived from each callus-clone for ploidy analysis. Distribution frequencies of DNA contents obtained via flow cytometry from wild cotton *G. trilobum*, Coker 312 and the somatic hybrids were 36, 70 and 100 respectively, indicating that the hybrids had a DNA content approximately equal to the sum of the two parents (Fig. 3).

Stomates

Observation of stomata on abaxial surface in the hybrids and parents revealed that the stomatal density in the hybrids was intermediate between the two parents. In addition, the stomates in the hybrids were considerably larger than those in either of the parent lines (Table 5; Fig. 4).

The deep-green hybrid leaves had more chloroplasts than the parent lines, with chlorophyll content in the hybrids of up to 2.16 mg/g fresh weight, 1.18 and 1.56 mg/g fresh weight in *G. trilobum* and Coker 312, respectively.

### Genomic analysis of somatic hybrid plants

RAPD assays of parental genomic DNA produced banding patterns characteristic of each species. Different banding patterns were obtained from 30 primer sets (Sangon listed, Shanghai). Two primer sets, S374 and S462, were selected for this analysis. The PCR products generated by S374 and S462 had characteristics of both parents (Fig. 5a, b) when hybrid plant material was used as template material, further indicating the plants regenerated from the clones were somatic hybrids. Some differences were observed in the banding patterns from each of the hybrids, indicating a rearrangement of the DNA material from both parents.

An SSR analysis of the two parents and seven of the somatic hybrid lines showed that the somatic hybrids contained the parental specific bands once again indicating their hybrid nature (Fig. 6).

SRAP analysis was carried out with the primer combinations of M1 to M8, paired with EM1 to EM10. The three combinations, M5 + EM5, M8 + EM10 and M7 + EM7, gave distinct patterns for the somatic hybrids and the two parents. Using the two combinations of M8 + EM10 and M7 + EM7, the somatic hybrid plants were shown to contain distinct bands of the two parents and were consequently confirmed as somatic hybrids (Fig. 7a, b).

AFLP analysis after *Eco*R I and *Mse* I digests showed that bands found in the two parents in addition to novel bands appeared in the somatic hybrids (Fig. 8a, b).

Together, the results of these four molecular markers techniques provide strong evidence that the somatic hybrids originated from the two parent lines.
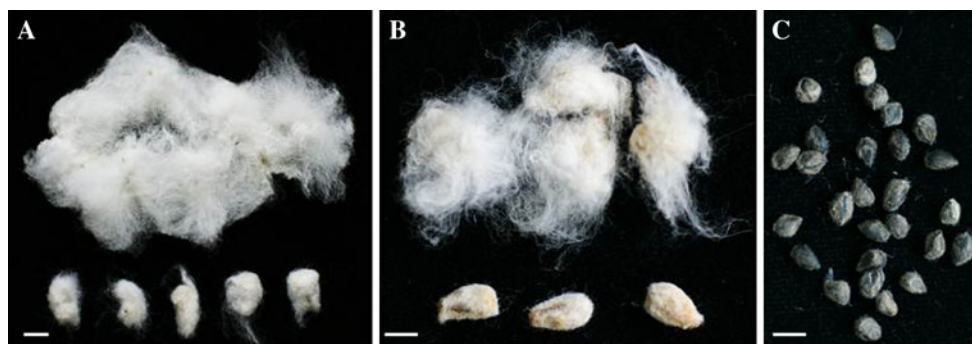
Fig. 2 The seeds and fiber of Coker 312, somatic hybrids and *G. trilobum*. **a** The seeds and white fiber of Coker 312, **b** the seeds and fiber of somatic hybrids, **c** the seeds and only fuzz of wild cotton *G. trilobum*. (*Bar* 5 mm)

Fig. 3 Flow cytometry analysis of the putative the somatic hybrid plants
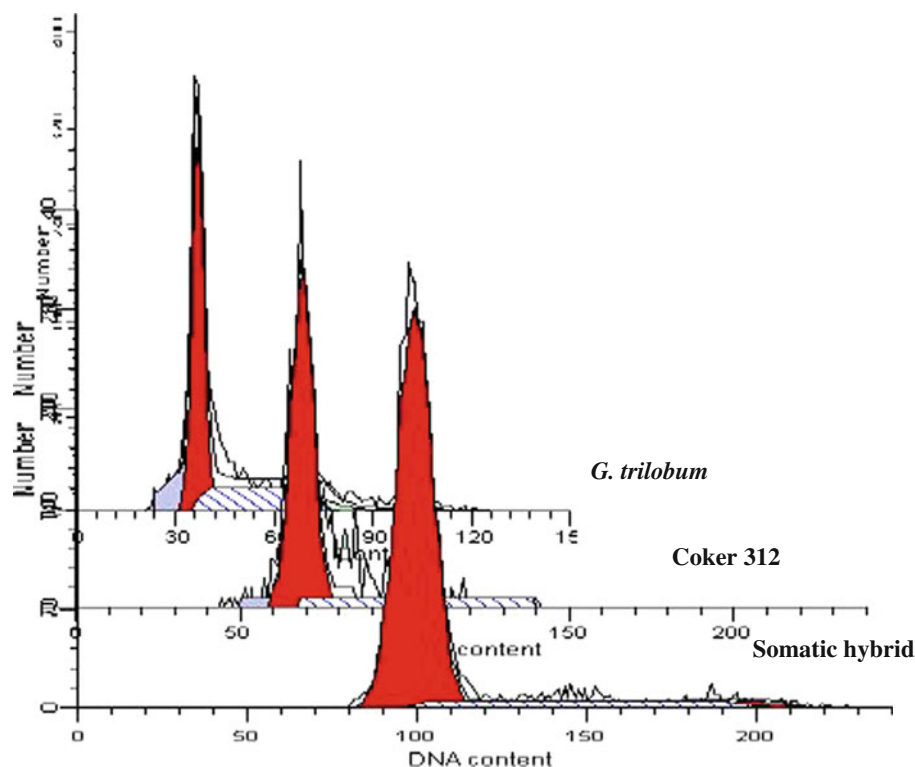
Table 5 Results of stomatal observation for the hybrid between Coker 312 and *G*

Species	Stomata density (No. mm^{-2})	Stomata length (μm)	Stomata width (μm)	Gland (No. mm^{-2})	Chlorophyll Content (mg/g)
G. trilobum	338.6 ± 67.7a	47.7 ± 5.1a	38.5 ± 1.3ab	11.4 ± 1.2a	1.18 ± 0.14a
Coker 312	422.2 ± 74.2b	47.0 ± 4.7a	35.8 ± 4.3a	14.5 ± 2.2b	1.56 ± 0.05b
Hybrids	346.5 ± 97.5a	49.2 ± 9.6a	43.3 ± 1.4b	18.8 ± 2.7c	2.16 ± 0.07c

Means followed by the same letters within a column do not differ significantly at $P < 0.05$ according to Duncan's multiple range test

In the mitochondrial and chloroplast analyses the somatic hybrids were shown to have similar banding patterns to the two parental plants, except for minor changes in lanes 7 and lane 9 (Fig. 9).

Analysis of photosynthesis in the somatic hybrids

Diurnal differences were observed in the rates of photosynthesis and transpiration and in stomatal conductance between the parent and somatic hybrid lines, however, the three characteristics were coincident at four time points 9 a.m. (dawn + 4 h), 12 a.m. (dawn + 7 h), 15 p.m. (dawn + 10 h) and 18 p.m. (dawn + 13 h). The photosynthetic and transpiration rates and stomatal conductance were lowest at 9 a.m. (dawn + 4 h) and reached a maximum at 12 a.m. (dawn + 7 h). They thereafter declined through 15 p.m. (dawn + 10 h) and 18 p.m. (dawn + 13 h) (Fig. 10). Interestingly, at all four time points, the photosynthetic rates, stomatal conductances and transpiration rates were all higher in the somatic hybrids than in the two parents. The three characteristics were lowest in the wild cotton *G. trilobum*, with intermediate characteristics in the upland cotton (Coker 312).

Analysis of gene expression for photosynthesis

In order to examine the basis for the increased photosynthetic rates in the somatic hybrids the expression of a number of photosynthesis-related genes were measured and compared between the parental and somatic hybrid lines.

Ten photosynthesis-related genes were analyzed (Fig. 11). Transcripts levels for *ndhF*, *ccsA*, *psaB* and *psaA*, were lowest in the wild cotton *G. trilobum* and were similar for *ycf3* and *psbA* in the upland cotton Coker 312 and *G. trilobum*. Transcript levels for all genes were highest in the somatic hybrids, consistent with the elevated
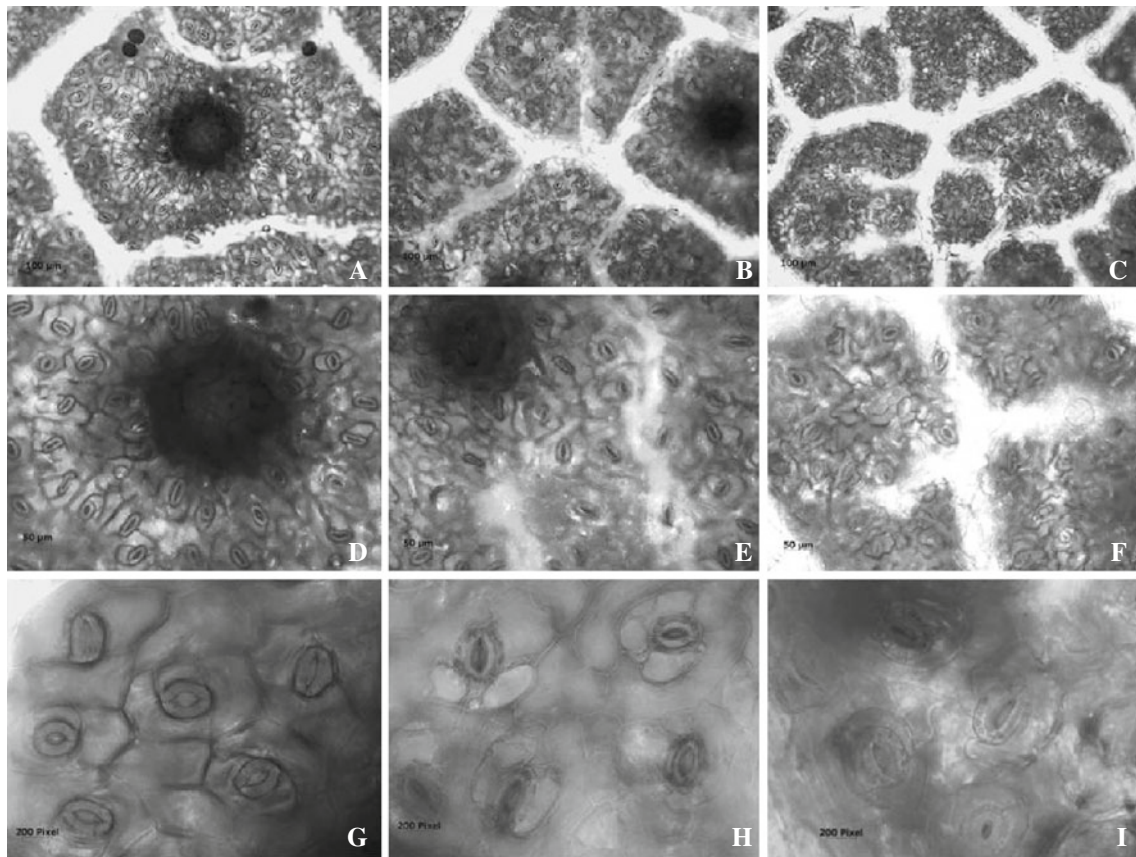
Fig. 4 Leaf epidermis stomatal morphology of somatic hybrids between Coker 312 and *G. trilobum*. **a**, **d**, **g** Upland cotton Coker 312; **b**, **e**, **h** somatic hybrids; **c**, **e**, **i** wild cotton *G. trilobum*. (**a**, **b**, **c**: ×200; **d**, **e**, **f**: ×400; **g**, **h**, **i**: ×1,000)
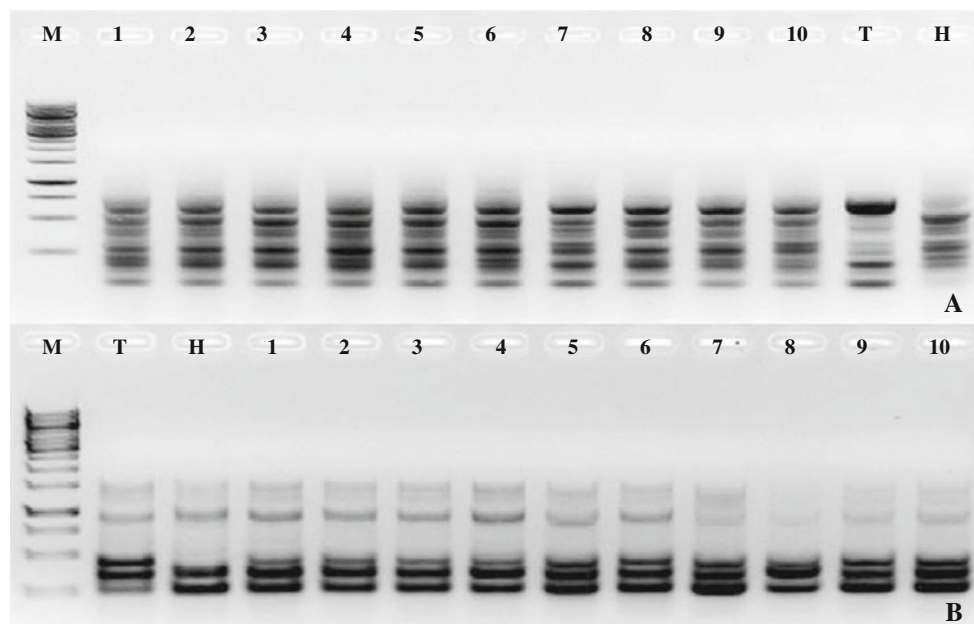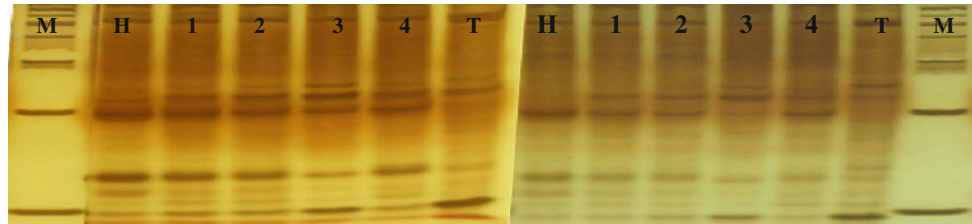

Fig. 5 RAPD analyses to identify somatic hybrids between Coker 312 and *G. trilobum*. M, DNA maker; T, *G. trilobum*; H, *G. hirsutum* cv. Coker 312; lane 1–10, somatic hybrids derived from each clone
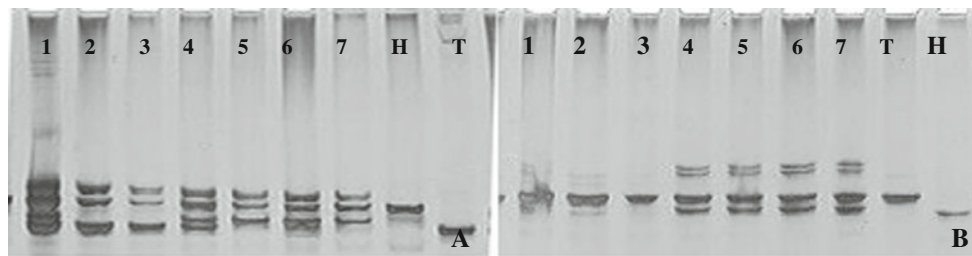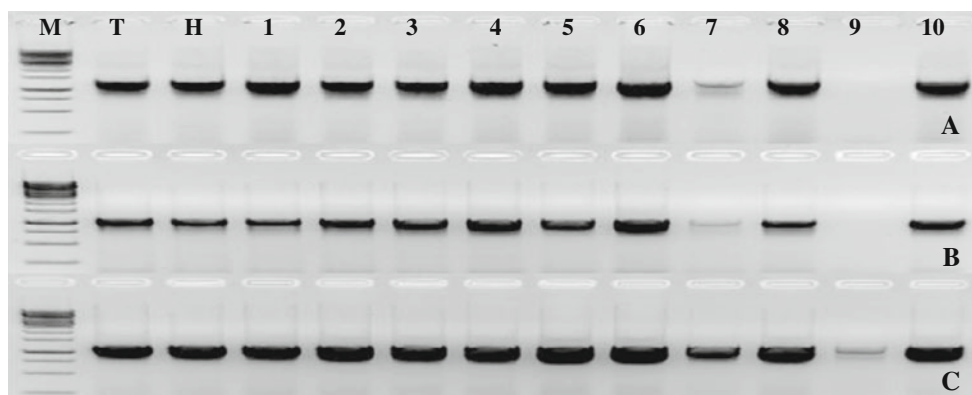
Fig. 6 SSR analyses to identify somatic hybrids between Coker 312 and *G. trilobum*. T, *G. trilobum*; H, Coker 312; lane 1–7, somatic hybrids derived from each clone
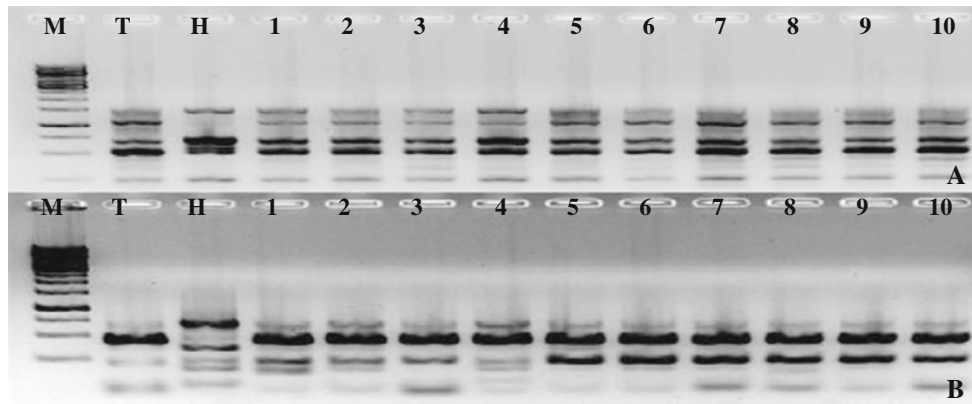

Fig. 7 SRAP analyses to identify somatic hybrids between Coker 312 and *G. trilobum*. M, DNA maker; T, *G. trilobum*; H, Coker 312; lane 1–10, somatic hybrids derived from each clone

Fig. 8 AFLP analyses to identify somatic hybrids between Coker 312 and *G. trilobum*. M, DNA maker; T, *G. trilobum*; H, Coker 312; lane 1–4, somatic hybrids derived from each clone

Fig. 9 Chloroplast and mitochondrial genomic analyses for somatic hybrids between Coker 312 and *G. trilobum*. **a**, **b** Chloroplast analysis; **c** mitochondrial analysis. M, DNA maker; T, *G. trilobum*; H, Coker 312; lane 1–10, somatic hybrids derived from each clone
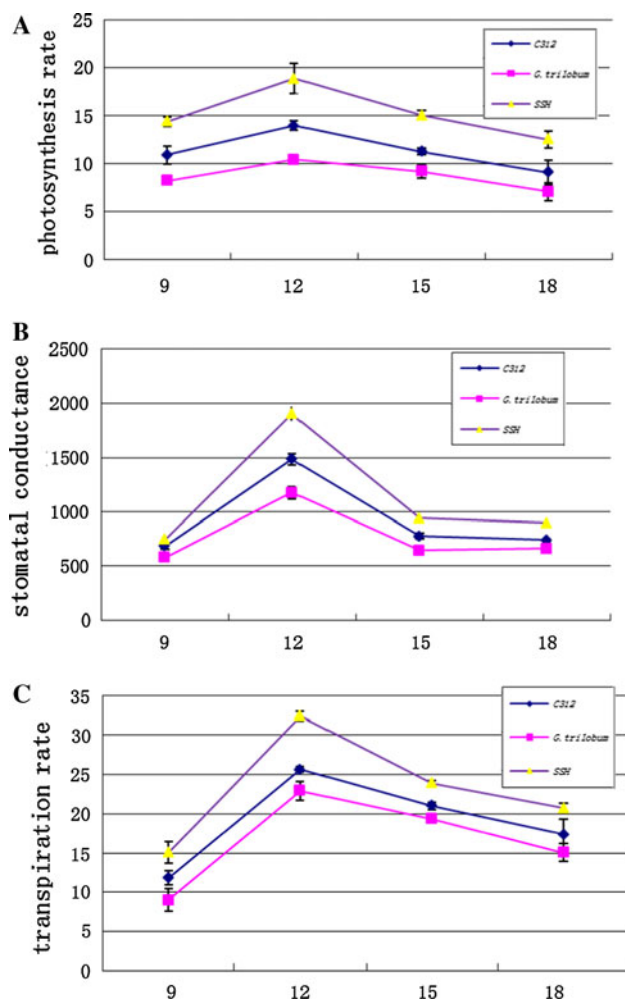
Fig. 10 Photosynthetic rate (**a**), stomatal conductances (**b**) and transpiration rate (**c**) of Coker 312, *G. trilobum* and somatic hybrids' leaves at different time of day on July 16 (9 a.m., 12 a.m., 15 p.m. and 18 p.m.)

level of photosynthesis in the somatic hybrids compared to the parent lines. It is possible that the photosynthetic phenotype is the result of a heterosis effect from the hybridization; however, similar effects were seen in subsequent generations, indicating that the effect may be the result of specific gene interactions rather than a general and transient epigenetic response.

The analysis of differential gene expression in somatic hybrids

In the small scale cDNA–AFLP analysis, a total of 104 bands were recognized as differentially expressed from the 60 amplicons (Fig. 12), indicating that the expression pattern of many genes was changed in the somatic hybrids compared to the parent lines. A more detailed expression analysis needs to be carried out in order to understand the extent and significance of the gene expression changes.

Discussion

The incorporation of diverse and divergent genomic material into upland cotton breeding programs is essential if breeders and growers are going to continue to meet the challenges that currently face the industry. Although conventional breeding programs have made steady improvements in agronomic traits, there is limited genetic diversity in the cultivated tetraploids, and this hampers the possibilities for further improvement (Kumria et al. 2003). More distantly related germplasms offer one avenue to broadening the available genetic diversity. However, because of genetic incompatibility and the reproductive isolation between cultivated varieties and wild cotton species, genetic improvement through interspecific hybridization is difficult and time consuming.

The results presented here clearly show that protoplast fusion is a feasible and efficient way to obtain somatic hybrids between sexually incompatible wild cotton species and elite upland cotton cultivars, become important additional approach to traditional methods for the creation of interspecific hybrids.

There are 51 species of cotton in the world. Of these, 46 are diploids with the genomes types, A–K and five are tetraploids with AD genomes (Fryxell 1992). *Gossypium arboreum* L., *G. herbaceum* L., *G. hirsutum* L., and *G. barbadense* L. are the only widely cultivated species. The upland cotton, *G. hirsutum* L., is the most important fiber crop. Many of the remaining species have recognized traits of interest to breeders. Because cultivated cotton varieties are highly susceptible to pests and diseases and abiotic stresses such as low temperature damage they require intensive crop management. The ability to efficiently create pest-, disease and cold-tolerant cotton varieties by incorporating useful traits from wild species would be of great value to breeders and growers alike. Many wild species have traits of considerable interest. For example, *G. anomalum* has recognized resistances to cotton bollworm, mite, leafhopper, drought, mildew-, and angular leaf spot. *G. thurberi* has been shown to be resistant to *verticillium wilt*, blight, and low temperature. The wild species, *G. trilobum*, has been shown to be highly resistant to *verticillium wilt*, blight and bacterial diseases, and is resistant to drought and cold temperatures of −7 to −10 °C. It also has the potentially useful trait of early abscission of leaves, which could be used to develop new varieties more amenable to mechanical harvesting.

Because the cultivated and wild cottons are sexually incompatible, biotechnological methods such as embryo rescue/ovule culture or interspecific cell fusion are required to overcome sexual barriers (Henn et al. 1998). Embryo rescue and ovule culture have been shown to be useful additions to cotton genetic improvement programs.

Fig. 11 Real-time PCR analyses of transcript levels for six selected genes about photosystem in two parental plants and somatic hybrids' leaves. Data represent fold change of gene expression in infected versus control samples. *Bars* represent a 95 % confidence interval, calculated on three technical replicates
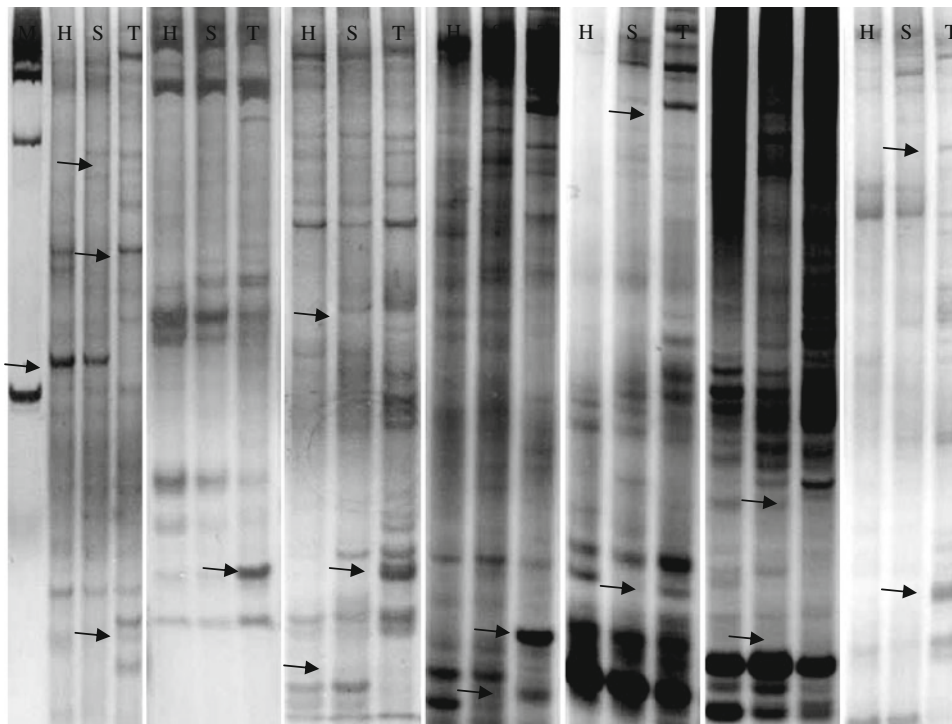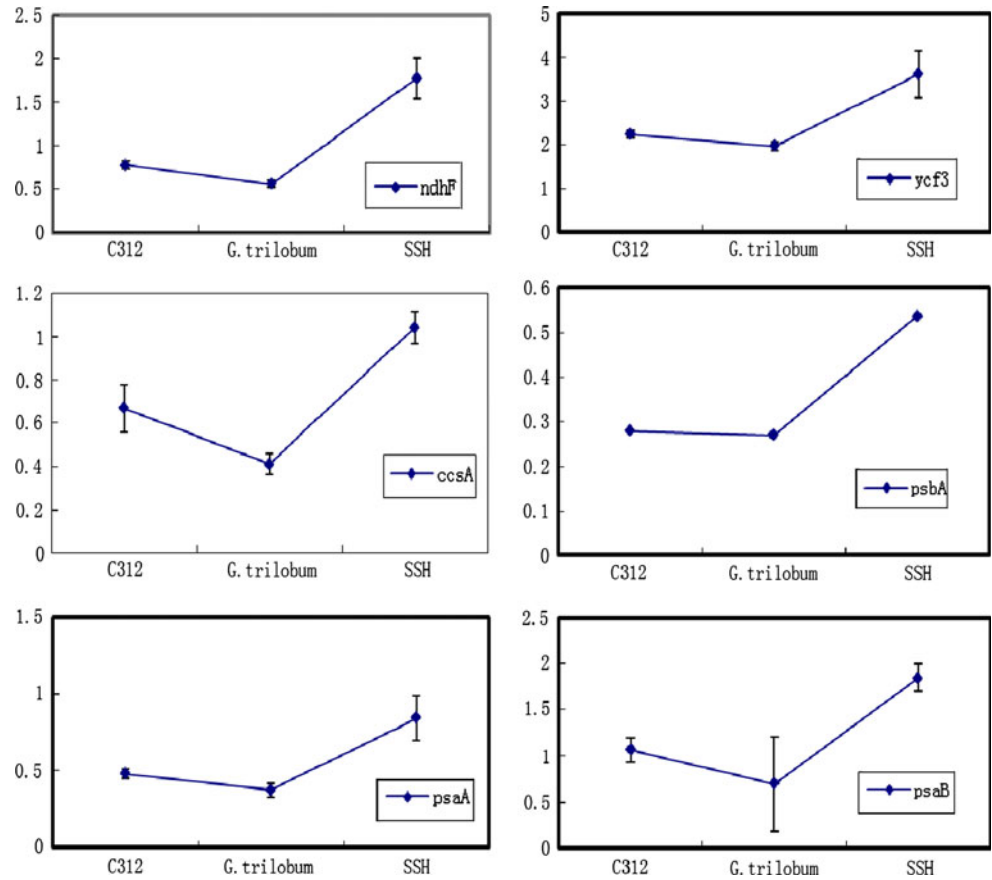
Fig. 12 Transcripts displayed by cDNA–AFLP of Coker 312, *G. trilobum* and their somatic hybrid. An example showing selective amplification with seven different primer combinations. M, DNA maker; T, *G. trilobum*; H, Coker 312; S, somatic hybrid

Somatic hybridization can also be used to overcome sexual crossing barriers and therefore potentially offers breeders the ability to introgress traits from wild species by combining genomes of incompatible species, or transferring nuclear and/or cytoplasmic traits (Atanassov et al. 1998; Cardi and Earle 1997). Biotechnological methods, as described in this work are an important additional approach for the creation of interspecific hybrids.

We have previously shown that the somatic hybridization technique can be used to successfully obtain symmetric somatic hybrids in cotton (Sun et al. 2004, 2005, 2011; Sun and Zhang 2006). Identification of the somatic hybrid status of the plants emerging from the hybridization is provided by morphological comparison, flow cytometry and a combination of molecular marker techniques. In the work presented here, hybridization between the tetraploid upland cotton *G. hirsutum* L. cv. Coker 312 and the wild cotton *G. trilobum* was verified initially by comparing the relative DNA content of the putative somatic hybrids. The relative DNA content of the hybrids was shown to be close to the sum of the DNA contents of the parents. Whereas the hybrid plants had an overall normal morphology and developmental timetable, they differed in several aspects from both parents. Particularly, stomates in the hybrids were much larger than those of the parents, leaf shape was altered in the hybrids and subsequently many intermediate materials of these hybrids will be created, reproductive development was somewhat perturbed. The somatic hybrid plants were also larger than the parent plants and had more chlorophyll and increased rates of photosynthesis. Differences from the parent material were also observed at the molecular scale. RAPD, SSR, SRAP and AFLP analyses of parental and somatic hybrid plants all revealed similar results, that the regenerated plants from fused protoplasts were somatic hybrids containing the genomes of both parents. The appearance of novel bands and the loss of some bands from the pattern observed in the parental lines indicated that the hybridization led to changes in genomic sequence order and/ or gene expression. The cDNA–AFLP results indicating changes in gene expression offers an explanation for the trait changes observed. Further analysis of the hybrids generated for this study will indicate whether the genetic and physiological traits are stable and of interest to cotton breeders. Intermediate materials such as those generated here have the potential to contain a number of novel traits that can be incorporated in breeding programs. It is possible, however, that the increased photosynthesis and robust nature of the hybrids presented here is due primarily to epigenetic changes as a result of heterosis (hybrid vigor). Further sexual crosses will reveal the genetic nature and utility of the novel traits. Regardless of this, however, the *G. hirsutum* and *G. trilobum* cross presented here is an important demonstration of the utility and potential for the method.

Incorporating *G. trilobum* genetic material is expected to open the possibilities for incorporating novel traits from the species, such as cold and drought tolerance, disease resistance and early leaf abscission, into upland cotton. The somatic hybrids generated here will be further examined for other novel traits of interest for use in the breeding of elite commercial cotton cultivars.

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