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Identification of a novel elite genotype for *in vitro* culture and genetic transformation of cotton

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Abstract

Hypocotyls of cotton (*Gossypium hirsutum* L.) cultivars cv. YZ-1, Coker 312 and Coker 201 were inoculated on Murashige and Skoog callus induction medium. YZ-1 exhibited a very high regeneration potential, with 81.9 % of the explants inoculated differentiated into embryogenic callus within 8 - 10 weeks. During the process of callus maintenance (subculture for 1 to 3 years), the total embryos number in Coker 312 and Coker 201 calli dropped sharply, and the percentage of embryo germination decreased. On the contrary, the callus of YZ-1 consistently maintains a high frequency of plant regeneration after long-time subculture. Transgenic kanamycin-resistant calli of Coker 201 partially lost the ability of somatic embryogenesis and plant regeneration. The stress produced by the transformation procedure slightly affected somatic embryogenesis and plant regeneration of YZ-1, which showed minimum loss of plant regeneration ability.

Additional key words: *Gossypium hirsutum*, regeneration potential, somatic embryogenesis.

Introduction

Cotton embryogenesis was first observed in *Gossypium koltzchianum*, but no plantlet regeneration was reported (Price and Smith 1979). Afterwards, plant regeneration was obtained from two-year old callus of *Gossypium hirsutum* L. cv. Coker 310 *via* somatic embryogenesis (Davidonis and Hamilton 1983). Since then, significant progress has been reported in cotton tissue culture (Finer and Smith 1984, Trolinder and Goodin 1987, Zhang *et al*. 1991, Firoozabady and DeBoer 1993, Wu *et al*. 2004, Aydin *et al*. 2004). At the same time, regeneration procedures have been used to obtain transgenic cotton *via Agrobacterium*-mediated transformation (Umbeck *et al*. 1987, Lyon *et al*. 1993) as well as by particle bombardment (Finer and McMullen 1990, Rajasekraran *et al*. 2000). Although research efforts targeting cotton have been ongoing for over a decade, there are less than twenty published reports on cotton transformation. One of the main reasons for this is the process of long-time tissue culture and transformation contributed to decline in vigor and regenerability of cultures, which includes low potency of embryogenesis, difficulty of embryos germination, and low rate of normal plantlets, as well as poorly developing roots, and low rate of success transplant in soil. The occurrence of loss of regenerability seems to be influenced by the plant genotype, explants type, the number of subcultures, and culture medium (Jain 2001). Lambe *et al*. (1997) proposed that the progress loss of totipotency in long-term tissue culture is a common event; which is a typical trait in plant neoplastic progression.

In addition, cotton regeneration *via* somatic embryogenesis is highly genotype-dependent. Among the Acala cotton cultivars tested, only few of them can regenerate (Mishra *et al*. 2003). Most lines developed from the cultivar Coker 312 (Trolinder and Xhixian 1989) were regenerable, but producers could not withstand the poor agronomic traits introgressed from Coker lines. Given the increasing percentage of transgenic cotton being grown worldwide, it is imperative to find more highly regenerable genotypes.

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Abbreviations: EC - embryogenic callus; MSB - MS medium (Murashige and Skoog 1962) and B5 (Gamborg *et al*. 1968) vitamins; IBA - indole-3-butyric acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA - α-naphthalene acetic acid; *npt* II - neomycin phosphotransferase Ⅱ.

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The objective of the research described herein was to identify new genotype, which exhibited higher regeneration potential than Coker 312 and Coker 201 and maintains it after long-term cultures (1 to 3 years). At the same time, our attention was focused on the analysis of the ability of somatic embryogenesis and plant regeneration of transgenic callus, which carries the *npt* Ⅱ gene, a popularly used gene for screening of transgenic callus in cotton.

Materials and methods

Cotton (*Gossypium hirsutum* L.) seeds of YZ-1, a cultivar of upland cotton developed by Mr. Qixiang Ma (Department of Plant Protection, Henan Academy of Agricultural Sciences, China) were used for this study, with two cultivars Coker 201 and Coker 312 (stored in our laboratory). Explants were prepared as previous described by Zhang *et al*. (1991). Seeds were decoated, sterilized with 0.1 % (m/v) $HgCl_2$ solution for 10 min and washed four times with sterilized distilled water. The sterile seeds were then inoculated on Murashige and Skoog (1962; MS) basal medium supplemented with 3 % (m/v) glucose, and solidified with 0.25 % (m/v) *Phytagel* (Sigma, St. Louis, USA) for germination. Seeds were cultured at 28 °C in the dark for 3 d and then transferred to the culture room (with temperature at 28 °C, a 14-h photoperiod, irradiance of 135 μmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps) for 4 d.

Hypocotyls were excised from aseptic seedlings and cut into 5 - 7 mm segments. Callus induction was carried out on MSB medium [MS inorganic salts and B_5 vitamins (Gamborg *et al*. 1968)] supplemented with 3 % (m/v) glucose, 0.25 % (m/v) *Phytagel*, 1.0 mg dm^{-3} indolebutyric acid (IBA), and 0.5 mg dm^{-3} kinetin for 4 - 6 weeks. A minimum of 80 explants (hypocotyls) per genotype was used for the research of regeneration potential, including at least 5 replicates. Calli characteristics were recorded at 3 and 6 weeks for colour, texture, dispersiveness in liquid, and cell or callus sizes.

The calli obtained were subcultured on a hormone-free MSB medium supplemented with 3 % (m/v) glucose and 0.25 % (m/v) *Phytagel* for the induction of embryogenic calli for 4 - 6 weeks. In this study, embryogenic calli (EC) were transferred on the MSB medium for embryo maturation, with KNO_3 was doubled but NH_4NO_3 removed, and supplemented with 3 % (m/v) glucose and 0.25 % (m/v) *Phytagel*, 0.5 mg dm^{-3} IBA, and 0.15 mg dm^{-3} kinetin, 1.0 g dm^{-3}glutamine, and 0.5 g dm^{-3} asparagines. All the media were autoclaved at 121 °C for 15 min except that the amino acids were filter-sterilized. Cultures were maintained in a room at 28 ± 2 °C under a 14-h photoperiod (irradiance of 135 μmol m^{-2} s^{-1}). EC were monthly subcultured on fresh medium for maintenance.

EC in 3.1-dm^3 flasks (200 ± 10 mg per flask) were cultured as one replicate and a minimum of 5 replicates were used for each genotype. The embryo numbers of calli with different age were recorded after 4 weeks of culture. The cotyledonary embryos (> 5 mm) were cultured onto basal MS medium in 100-cm^3 flasks for plant regeneration, and the percentage of mature embryos germination was recorded 4 weeks after culture. The regenerated plantlets with true leaves and good roots were hardened and transferred to the greenhouse.

At the same time, the survey was also focused on the effect of stress coming from the transformation protocols *via Agrobacterium tumefaciens*. Newly differentiated EC (subcultured less than one year) were divided into two parts: one part was used for transformation, the other was used as control maintained on MSB medium by monthly subculturing to fresh medium. All plant transformation and regeneration procedure were completed according to the method described by our previous work (Chen *et al*. 2001, Jin *et al*. 2005). The *Agrobacterium* strain LBA4404 harbouring *pΔgusBin19* construct (Topping and Lindsey 1991) were used for delivery of kanamycin resistance. To verify the presence of introduced *npt*-II gene in kanamycin-resistant calli, genomic DNA was extracted as our previous report (Jin *et al*. 2005) and PCR was conducted by using the primers P1:
5'- TCCGGCGCTTGGGTGGAGAG- 3' and P2:
5'- CTGGCGCGAGCCCCTGATGCT- 3' for amplifying a 470-bp coding-region of *npt*-II gene. PCR amplification conditions consisted of denaturation at 94 °C, 2 min; annealing at 63 °C, 1 min; extension at 72 °C, 1 min (totally 30 cycles); and finally extension at 72 °C, 10 min. PCR positive calli were further tested by Southern blot hybridization using the standard methods of Sambrook *et al*. (1989). DNA preparations were digested with *Hind*Ⅲ and *Sst*I to release a 1500 bp fragment containing the *npt*-II. ^{32}P-labeled probes were prepared using the coding region of the *npt*-Ⅱ gene from the PCR product.

Five replicates, consisting of 10 flasks containing approximately 1.5 g transformed EC (30 d after subculture) of YZ-1 and Coker 201 were prepared. The embryo numbers of calli and the percentage of mature embryos germination were recorded according to the same method described previously. Significant results were determined by analysis of variance (*ANOVA*) and the differences between the means were compared by the least significant differences (LSD).

Results

Highly friable yellowish-white calli were capable of somatic embryogenesis (Fig. 1*A*). In contrast, hard, dark green, non-friable calli did not differentiate into embryogenic calli (Fig. 1*B*). Callus induction efficiency was evaluated based on four criteria: colour, texture, dispersiveness in liquid media, and size/shape of undifferentiated cells as defined by Mishra *et al.* (2003). Explants of the three genotypes produced calli on MSB callus induction medium in great quantities. Of the inoculated explants of YZ-1 81.9 % differentiated into embryogenic cultures 6 weeks after subculturing onto MSB medium (Table 1), while only 60.5 and 68.3 % of Coker 201 and Coker 312, respectively. The result means that high frequency of somatic embryogenesis of YZ-1 can be obtained within 10 - 12 weeks.

Callus age showed a strong effect on the capacity

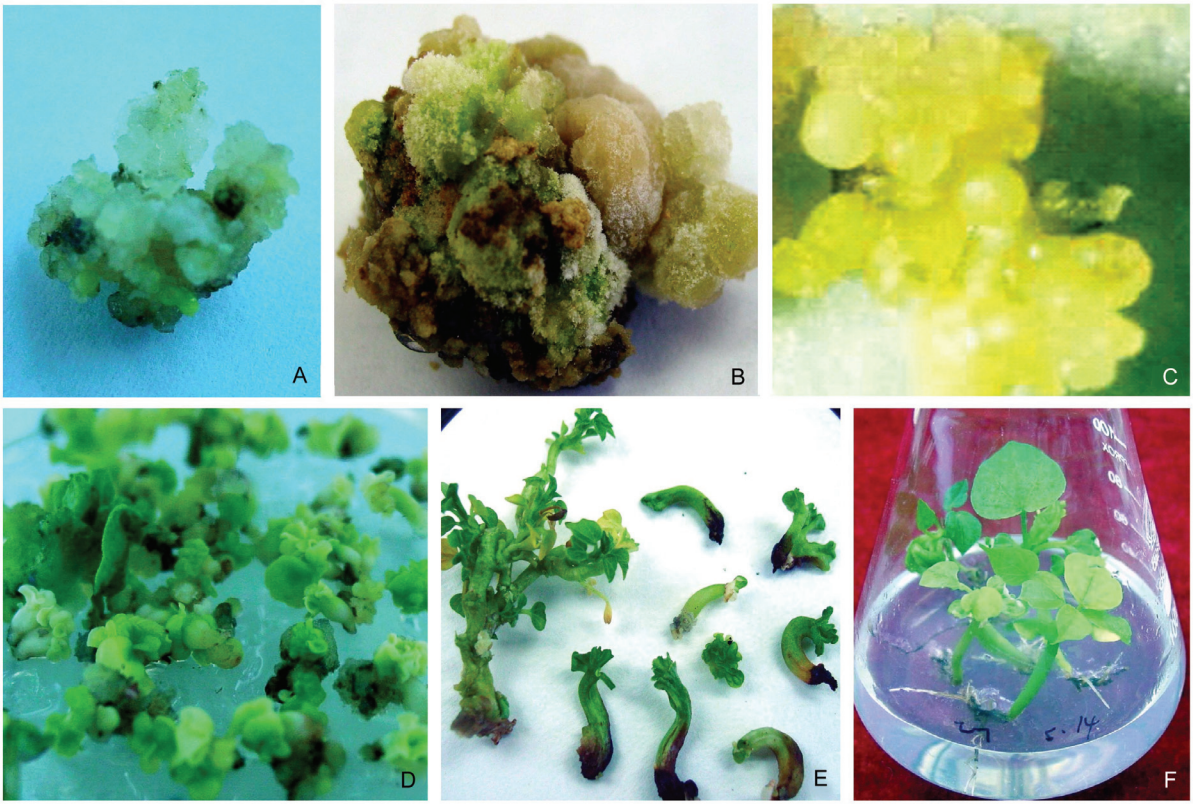

Fig. 1. Somatic embryogenesis and plant regeneration in cotton: *A* - friable, yellowish callus produced from explants of YZ-1 6 weeks after inoculation; *B* - hard, dark-green, compact callus produced by Coker 312 at the same time as YZ-1; *C* - globular stage embryos in Coker 312 calli. *D* - cotyledonary stage embryos in YZ -1 calli. *E* - different kinds of abnormal plantlets initiated from aged calli of Coker cottons; *F* - normal plantlets initiated from 3-year-old calli of YZ-1.

of plant regeneration. Young calli (less than one year) of three genotypes gave a large number of globular- and cotyledon-stage embryos, and produced many normal plants because of a high percentage of embryo germination. Unfortunately, with the aging of calli, the embryo number in Coker 201 and Coker 312 calli dropped sharply, especially the percentage of embryo germination showed a rapid decrease (Table 2), which explain why plant regeneration and transformation need young calli. To our surprise, the callus of YZ-1 consistently maintained high efficiency of plant regenerability after long-time subculture. Especially, the percentage of embryo germination was comparatively high, which produced numerous normal plants within short time (Table 2, Fig. 1*F*). Although the formation of globular embryos was high in Coker 312 and Coker 201 calli (Fig. 1*C*), only 3 to 14 embryos in 1 g EC developed into the cotyledonary stage. Furthermore, a high proportion of cotyledon-stage embryos showed various degrees of abnormalities, such as multiple cotyledons, lack of shoot tip, and fused cotyledons (Fig. 1*E*). However, the number of cotyledon-stage embryo in YZ-1 is much higher (2 to 5 times) than that of Coker 201 and Coker 312 callus in different ages (1 to 3 years) (Fig. 1*D*, Table 2), and much more cotyledon-stage embryos are normal in morphology (Fig. 1*D*,*F*).

Putatively transformed calli were analyzed by PCR with specific primers of selection marker gene *npt*-II.

Table 1. Regeneration potential of three cotton (*Gossypium hirsutum* L.) cultivars (percentage of explants producing callus 4 - 6 weeks after initiation and percentage of calli producing somatic embryos 6 weeks after subculturing onto embryo induction medium. Means followed by the same letters within a column do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test.

Genotypes	Number of explants	Callus production [%]	Embryogenetic potential [%]
YZ-1	108	95 ± 1.8a	81.9 ± 2.5a
Coker 201	87	91 ± 2.3a	60.5 ± 2.7b
Coker 312	81	92 ± 1.2a	68.3 ± 0.8b

Table 2. Effects of calli age on plant regeneration ability (number of globular-stage embryos per gram callus, 30 d after subculture; number of cotyledon-stage embryos per gram callus, 30 d after subculture; percentage of germination = number of normal plants (with true leaves and good roots)/number of cotyledonary-stage embryos inoculated). Means followed by the same letters within a column do not differ significantly at $P \leq 0.05$ according to Duncan's multiple range test.

Genotypes	Age [year]	Globular stage	Cotyledonary stage	Embryo germination
YZ-1	<1	260.0 ± 7.9a	29.4 ± 3.1a	36.6 ± 3.6a
	2	231.3 ± 3.9a	17.4 ± 2.9b	24.2 ± 3.5b
	3	246.1 ± 6.4a	16.1 ± 4.0b	21.0 ± 3.3b
Coker 201	<1	188.3 ± 10.8b	14.7 ± 1.9b	26.7 ± 2.6b
	2	97.7 ± 4.6c	6.8 ± 1.8c	7.3 ± 0.9c
	3	63.5 ± 7.8d	2.3 ± 1.2d	1.7 ± 0.8d
Coker 312	<1	197.5 ± 7.5b	11.9 ± 1.3b	23.5 ± 0.6b
	2	105.6 ± 3.6c	4.9 ± 1.2c	5.9 ± 0.7c

Five independent kanamycin-resistant calli were identified to contain the correct *npt*-II transgenes (Fig. 2). Genomic DNA from these calli plants were subjected to further analysis using Southern blot hybridization (Fig. 3). Transformants genomic DNA cut with *Hind*III-*Sst*I and probed with *npt*-II resulted in the predicted 1500 bp hybridization fragment.

The PCR and Southern blotting confirmed transgenic calli of Coker 201 and YZ-1 were used for comparison of the plant regenerability of transgenic clones. The number of globular and cotyledonary stage embryos of Coker 201 transgenic callus dropped dramatically after exposure to kanamycin. The same situation can also be seen in plant regeneration. Only 13.1 % cotyledon-stage embryos can develop into normal plants (Table 3). However, transgenic callus of YZ-1 showed a small decline in the embryos production and percentage of germination.

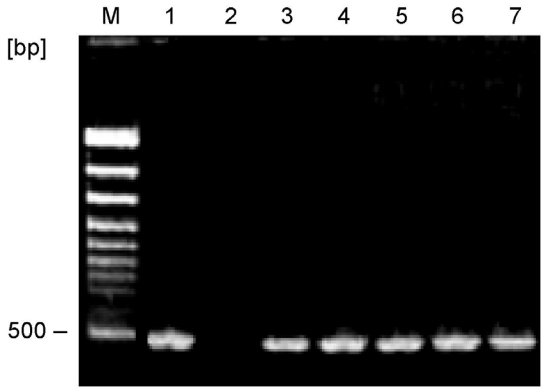

Fig. 2. PCR analysis of genomic DNA to detect the presence of the *npt*-II coding region. *Lanes*: M - molecular mass markers; 1 - plasmid of pΔgusBin19; 2 - untransformed control calli; 3 - 7 - independent kanamycin-resistant calli showing amplification of the predicted 470 bp *npt*-II gene specific fragments.

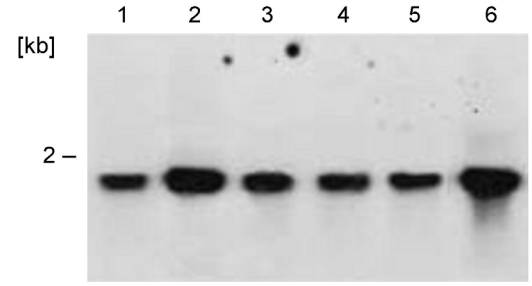

Fig. 3. Southern blot analysis of transgenic calli. *Lane* 1: genomic DNA from plasmid pΔgusBin19; *lanes* 2 - 6: genomic DNA of PCR positive independent kanamycin-resistant calli were digested with *Hind*III-*Sst*I and hybridized with the *npt*-II probe prepared from the plasmid by PCR.

Table 3. Comparison of the plant regeneration ability of control (C) and transgenic (T) calli, see Table 2 for detail.

Genotypes	Type of callus	Globular stage	Cotyledonary stage	Embryo germination
YZ-1	C	258.3 ± 14.5a	17.3 ± 1.3a	37.5 ± 3.1a
	T	245.5 ± 17.8a	13.5 ± 1.8b	30.1 ± 2.2b
Coker 201	C	186.0 ± 10.5b	14.8 ± 2.5b	21.3 ± 2.5c
	T	147.0 ± 14.2c	6.0 ± 2.3c	13.1 ± 1.8d

Discussion

Although somatic embryogenesis is comparatively easy for Coker lines, cotton still remains one of the recalcitrant species to manipulate in *in vitro* culture (Wilkins *et al.* 2000). The differential induction of embryogenic calli by Coker 201, Coker 312 and YZ-1 explants reflected inherent genetic differences in accordance with the results

recorded by Firoozabady and DeBoer (1993) and Nobre *et al*. (2001). Genotype-dependence could be overcome in different degrees through optimizing culture factors as our previous report (Wu *et al*. 2004), but it is still a key factor for somatic embryogenesis and plant regeneration in cotton.

Long culture times significantly contribute to the decline in vigour and regenerability of Coker cultures (Table 2). The loss of culture health with time is major detriment to the efficiency of plant regeneration and transgenic plant production. The genotype plays a major role in decreasing the regenerability of cultures (Anu *et al*. 2004), so, the genotype YZ-1 described here is very useful for improving the efficiency of cotton tissue culture and transformation, which was proved by the fact that we have obtained considerable transgenic plants of YZ-1 within short time despite the long-time culture.

EC used as explants for transformation has been reported by many authors (Hiei *et al*. 1997, Cheng *et al*. 1997) in other crops. Our laboratory (Chen *et al*. 2001, Jin *et al*. 2005) developed the similar transformation procedure in cotton with *Agrobacterium tumefaciens* by using EC as infected explants. Transformation protocols include the use of many components that potentially provide a stress to the cells grown in culture (Bardini *et al*. 2003). It has been shown that β-lactam antibiotics interfere with growth, rooting and shoot regeneration of cultured cells and tissues (Montserrat *et al*. 2001), and that kanamycin causes hypermethylation in tobacco plants (Schmitt *et al*. 1997), DNA methylation changes in callus of *Arabidopsis thaliana* (Bardini 2003). So, loss of plant regeneration ability becomes problematic with such long culturing times and the stress provided by the antibiotics. It is well known that minimum concentration of antibiotics and a minimum time spent in selection remarkably decreased regenerability (Biotel-Conti *et al*. 2000, Bardini *et al*. 2003), but difference existed among genotypes (Wang *et al*. 2000). YZ-1, described here, showed an enhanced regeneration potential under the stress of transformation. The higher tolerance of YZ-1 to the stress produced by transformation procedure has virtually eliminated the loss of regeneability.

This study successfully increased the range of cotton genotypes that can be regenerated, and saved the regeneration time as much as 4 - 5 months. The highly regenerable YZ-1 germplasm described in this study has been successfully transformed (Jin *et al*. 2005), and provided an avenue for direct introduction of novel traits into cotton *via Agrobacterium* in breeding programs that may ultimately expedite cultivar development.

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