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Highly efficient plant regeneration through somatic embryogenesis in 20 elite commercial cotton (*Gossypium hirsutum* L.) cultivars

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Abstract

A protocol was established for cotton somatic embryogenesis and plant regeneration. Using this protocol, highly efficient plant regeneration via somatic embryogenesis was obtained from more than 20 Chinese and Australian commercialized cotton cultivars including CCRI 12, CCRI 19, and Simian No 3. These three cultivars alone comprise more than 50% of the total cultivated cotton in China. Based on three criteria, the 20 tested cotton cultivars were classified into three different groups: easily embryogenesis-induced cultivars (such as CCRI 19, Simian No 3, Lumian 6, Sikral 1-3, Coker 201), moderately easy embryogenesis-induced cultivars (such as CCRI 16, CCRI 24, Simian No 4 and CCRI 29), and not easily embryogenesis-induced cultivars (such as CCRI 17, CCRI 30 and CCRI 27). The three criteria used included (1) the somatic embryogenesis ratios, (2) the time required to produce somatic embryogenesis, and (3) the number of the somatic embryos produced in each culture. Also, the plant hormone Zeatin (ZT) was used to induce direct somatic embryogenesis and had the highest induction ratio of 33.3%. Overall, this procedure simplifies cotton somatic embryogenesis from a multi-step culture process to a one-step culture process and shortens the culture cycle from 180 to 60-120 days. This protocol also makes it easier to control the somaclonal variation in plant tissue culture and facilitates the application of plant genetic engineering on cotton genetic improvement.

Keywords: cotton, somatic embryogenesis, genotype-independent, dehydration, plant regeneration, plant growth regulator, hormone

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 2iP-6-γ,γ-dimethyl-allyamino-purine; ABA, Abscisic acid; B_5, Gamborg (1968) medium; BA, benzyladenine; BR, Brassinolide; GA, Gibberellic acid 3; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KT, kinetin; MS, Murashige and Skoog (1962) medium; MSB, a medium consisting of organic macro and microelements of MS medium and vitamins and amino acids of B_5 medium; NAA, α-naphthaleneasetic acid; TDZ, thidiazuron; ZT, zeatin.

Introduction

Cotton is one of the most important economic and fiber crops. It is cultivated in 70 tropical/subtropical and temperate countries around the world with the United States, China, India, and Australia leading the world in cotton production. The cultivation of cotton has affected the economic development of numerous countries since the plant was first cultivated around 5,000 to 10,000 years ago (Stephens and Mosley 1974). Over 180 million people are associated with the global fiber industry, which annually produces 20 to 30 billion dollars worth of raw cotton (ICAC 1996). Trade in cotton-related products amounts to

about 50% of the total global trade in textiles, producing $115 billion, and in clothing, producing $133 billion (Rabobank 1996).

Ever since the first transgenic cotton plant was produced in 1990, tissue culture-based technologies have provided important contributions for plant regeneration (Wilkins, et al. 2000; Zhang, et al. 2000; Zhang, et al. 2005), not only for conventional breeding but also for the direct genetic improvement of cotton. In the middle of the 1990s, transgenic cotton became one of the first successful commercialized transgenic plants and in 2008 approximately 15.5 million hectares of transgenic cotton were planted, which accounts for 12% of the total cotton planted around the world. (James 2008).

Although transgenic cotton has been rapidly adopted for commercial use over the past 10 years, obtaining transgenic cotton cultivars has been difficult. While transgenic technology has attempted to transfer desirable genes into cotton cells, this process has been followed by the difficult task of plant regeneration. Until now, only a few cotton cultivars have been found to be capable of somatic embryogenesis and plant regeneration and even so, most of these regenerable cultivars are ones that are no longer used in the field. Consequently, almost all transgenic cotton cultivars have been obtained by cross breeding after obtaining transgenic plants, an approach which is time consuming and expensive (Perlak, et al. 2001; Zhang, et al. 2005).

Although regeneration efficiency via somatic embryogenesis has been improved in cotton in the past 20 years (Aydin, et al. 2006; Divya, et al. 2008; Han, et al. 2009; Hussain, et al. 2009; Khan, et al. 2006; Kouakou, et al. 2007; Michel, et al. 2008; Sun, et al. 2006; Wang, et al. 2008), genotype-dependent responses and prolonged culture periods still remain two of the major problems associated with cotton regeneration. In this study, we first attempted to investigate the effect of different factors (genotypes, explants, and plant growth regulators) on cotton somatic embryogenesis and plant regeneration. Next, we developed a simple procedure for highly efficient and rapid plant regeneration via somatic embryogenesis for current commercialized cotton cultivars.

Materials and methods

Plant materials and the establishment of sterile seedlings

Seeds of the Chinese commercialized cotton (*Gossypium hirsutum* L.) cultivars CCRI 12, CCRI-13, CCRI 16, CCRI 17, CCRI 19, CCRI 24, CCRI 27, CCRI 29, CCRI 30, Simian No 3, Simian No 4, Lumian 1, Lumian 6, Jinmian 7, Jinmian 12, Jihe 321, Yumian 8; the Australia commercialized cotton cultivars Siokral 1-3 and Siokral 1-4; and the elite regenerable cotton cultivars Coker 201 and Coker 312 were obtained from the National Cotton Germplasm Facility, Cotton Research Institute, The Chinese Academy of Agricultural Science, Anyang, Henan, China.

Kernels were removed from the seeds. Mature kernels were chosen and were surface-sterilized with 0.1% $HgCl_2$ for 7 minutes, and then rinsed three times with sterile distilled water. The kernels were then cultured on a 0.7% agar-solidified medium, pH 5.8, that contained half-strength Murashige and Skoog (MS) inorganic salts (Murashige and Skoog 1962), B_5 vitamins (Gamborg, et al. 1968), and 2% sucrose for germination. The kernels were cultured at 28°C ± 2°C under 16 h light photoperiod conditions with a light intensity of approximately 24 PPFD. All chemicals, including all plant growth regulators, were purchased from Beijing Biochemical Company (Beijing, China).

Induction of callus

Hypocotyl sections (3-5 mm length), cotyledon pieces (10~16 mm^2 surface area), and root segments (3-5 mm length) of 7-day-old sterile seedlings were cultured on basic MS medium supplemented with various concentrations (0-3.0 mg/L) of plant growth regulators (ZT, KT, 2,4-D, and 2iP) for inducing callus.

Selection of high frequency embryogenic cell lines

After 60-120 days of culture, embryogenic callus was chosen and transferred onto MSB medium (MS inorganic salts and B_5 vitamins) supplemented with different hormones (0.1-3.0 mg/L ZT, and 0.1-1.0 mg/L KT, IAA and 2,4-D) or onto MSB medium without hormones for the proliferation of embryogenic callus. After 28 days of subculture, embryogenic callus with a high frequency of embryogenesis was chosen for the next subculture. The remaining subcultures were carried out in the same way. Subsequently, embryogenic callus was subcultured every 28 days on MSB medium supplemented with 0.1 mg/L ZT (Zeatin) and 2 g/L activated charcoal. Based on our previous study, 2 g/L of activated charcoal significantly enhanced the proliferation and differentiation of cotton embryogenic callus.

Table 1. Comparison of somatic embryogenesis among genotypes and the nature of explants using different ZT concentrations *

Hormones (mg/L)	Explants	CCRI 12			CCRI 19			Simian No 3		
		No. of explants	% Callus induction	embryogenic callus %	No. of explants	% Callus induction	embryogenic callus %	No. of explants	% Callus induction	embryogenic callus %
0.1 ZT	Hypocotyl	55	98.2	3.64	60	96.7	1.76	18	100.0	5.56
	Cotyledon	35	48.6	11.42	59	44.1	6.78	33	21.2	9.09
	Root	23	21.7	8.70	51	19.6	3.92	25	64.0	8.00
0.5 ZT	Hypocotyl	72	97.2	0.00	48	97.9	0.00	27	100.0	0.00
	Cotyledon	53	17.0	7.55	52	57.7	0.00	22	63.6	4.55
	Root	26	11.5	3.85	33	24.2	0.00	8	25.0	0.00
1.0 ZT	Hypocotyl	50	100.0	0.00	N.A.	N.A.	N.A.	38	100.0	0.00
	Cotyledon	66	28.8	0.00	N.A.	N.A.	N.A.	36	2.8	0.00
	Root	28	67.9	0.00	N.A.	N.A.	N.A.	19	100.0	0.00
3.0 ZT	Hypocotyl	45	100.0	0.00	N.A.	N.A.	N.A.	42	100.0	0.00
	Cotyledon	52	96.2	0.00	N.A.	N.A.	N.A.	40	27.5	0.00
	Root	27	74.1	0.00	N.A.	N.A.	N.A.	21	100.0	0.00
0.1 2iP	Hypocotyl	36	88.9	0.00	68	88.2	0.00	48	97.9	0.00
	Cotyledon	48	58.3	0.00	76	47.4	2.63	60	68.3	3.33
	Root	28	21.4	0.00	33	33.3	0.00	30	63.3	0.00
0.1 ZT + 0.1 2,4-D	Hypocotyl	76	100.0	0.00	56	100.0	0.00	48	100.0	0.00
	Cotyledon	84	100.0	0.00	58	100.0	0.00	48	100.0	0.00
	Root	54	100.0	0.00	48	100.0	0.00	36	100.0	0.00
0.5 ZT + 0.1 2,4-D	Hypocotyl	60	100.0	0.00	46	100.0	0.00	48	100.0	0.00
	Cotyledon	86	96.5	0.00	58	100.0	0.00	48	100.0	0.00
	Root	50	100.0	0.00	52	100.0	0.00	38	100.0	0.00
0.1 ZT + 0.1 IAA	Hypocotyl	65	100.0	0.00	60	98.3	0.00	40	100.0	0.00
	Cotyledon	39	43.6	5.13	63	39.7	3.17	49	36.7	0.00
	Root	30	18.2	0.00	48	14.6	0.00	32	53.1	0.00

* N.A.: no data available for this study because no test was performed for this cultivar.

Differentiation of somatic embryos and plant regeneration

High frequency embryogenic cell lines were chosen and transferred onto embryo differentiation medium (MSB medium supplemented with 0.1 mg/L ZT) for the induction and development of somatic embryos. Mature embryos were chosen and transferred onto embryo germination medium (MSB or ZH (Zhang, et al. 1996) medium supplemented with 0.1 mg/L ZT and 2 g/L activated charcoal) after 30 days.

All media were supplemented with 30 g/L sucrose, and were solidified with 7 g/L agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 minutes. All cultures were incubated at 28°C ± 2°C under a 16 h light photoperiod with a light intensity of approximately 24 PPFD, which was provided by cool white fluorescent lamps.

Experimental setup and statistical analysis

All experiments were repeated approximately 3-6 times. All obtained data was processed by statistical analysis using standard statistical software (SPSS, Chicago, Illinois, USA). A probability level of 5% (=0.05) was chosen for all statistical inferences. The analysis of variance and the Least Significant Difference (LSD) methods were used to detect differences among treatments.

Results

Induction of embryogenic callus

After 5-7 days of culture, callus started to appear at the surface of cut explants. The formation of callus, however, is influenced by many factors including plant growth regulators, genotypes, types of explant, and the components in the medium. After 30 days of culture, two types of callus (embryogenic and non-embryogenic callus) were observed from different culture events (Fig. 1 a-c). Direct somatic embryogenesis was also observed from cultured hypocotyls, cotyledons, and root explants (Fig. 1 c and d). During cotton cell undifferentiation and differentiation, plant growth regulators played a critical role. Generally speaking, ZT was a good plant regulator for inducing direct embryogenesis but not a good regulator for the rapid proliferation of callus. In contrast, 2,4-D promoted the formation of non-embryogenic callus with high frequency and large mass, but it was difficult to induce embryogenesis by using 2,4-D.

In previous studies, we found that medium only containing ZT induced the formation of embryogenic callus and for direct cotton somatic embryogenesis, the best concentration of ZT was 0.1 mg/L. (Zhang, et al. 2001; Zhang, et al. 1999; Zhang, et al. 2001). In this study, we chose 3 major Chinese comercialized cotton cultivars, which account for more than

Table 2. Somatic embryogenesis of 20 Chinese and Australian commercial cotton cultivars

Genotype	No of explants	% Callus induction	% Embryogenic callus induction	Days required for obtaining embryogenesis	Number of visible mature embryos
CCRI 12	35	48.6	11.4±2.7	80	5.0±1.2
CCRI 13	105	56.2	8.6±3.8	40	4.8±1.6
CCRI 16	96	50.0	3.1±0.0	99	3.2±1.1
CCRI 17	87	33.3	1.1±0.4	102	0.0±0.0
CCRI 19	59	44.1	6.8±2.5	29	10.5±3.5
CCRI 24	102	49.0	3.9±2.0	87	4.8±1.6
CCRI 27	113	51.3	8.0±2.6	109	0.0±0.0
CCRI 29	97	50.5	4.1±2.2	78	2.1±0.7
CCRI 30	97	47.5	1.0±0.3	112	0.0±0.0
Simian No 3	33	21.2	9.1±1.9	32	9.2±0.3
Simian No 4	110	53.6	4.5±2.1	58	6.2±2.1
Lumian 1	101	25.7	5.9±2.8	78	3.9±1.3
Lumian 6	100	36.0	9.0±3.7	39	7.8±2.9
Jinmian 7	108	30.6	9.3±1.2	35	6.0±2.1
Jinmian 12	99	48.5	2.0±0.9	97	1.2±0.7
Jihe 321	110	50.9	7.3±1.3	35	6.9±3.5
Yumian 8	95	40.0	1.0±0.3	109	0.0±0.0
Siokral 1-3	96	71.2	16.7±0.7	33	8.9±2.1
Siokral 1-4	102	56.9	4.9±1.5	48	6.3±2.0
Coker 201	108	66.7	33.3±0.9	23	18.9±1.6
Coker 312	99	51.5	20.2±1.3	29	13.6±2.5

Medium is MSB with 0.1 mg/L ZT. Values represent means ± SD. The embryogenesis ratios and somatic embryos were observed 120 days after culture. Ratios of somatic embryogenesis were calculated as the number of explants with embryogenic callus compared to the number of cultured explants. Number of visible mature embryos was calculated as the number of observed mature embryos (torpedo-shaped embryos and cotyledon embryos) compared to the number of explants with embryogenic callus.

50% of the total cotton cultivated in China, to test the role of ZT in cotton somatic embryogenesis. Similar results were observed as in previous experiments (Table 1). These results, added to the data from the previous studies, indicate that the plant regulator ZT could directly induce cotton somatic embryogenesis (Fig.1 c and d).

Significant differences were observed when inducing direct cotton somatic embryogenesis using different plant growth regulators. In this study, we observed that only ZT and 2iP induced direct somatic embryogenesis from cotton explants. We also observed that it was easier to induce cotton somatic embryogenesis using ZT rather than 2iP. In this experiment, 2iP only induced cotyledon explants to produce embryogenic callus from CCRI 19 and Simian No 3 but not from CCRI 12. ZT induced somatic embryogenesis with a high frequency in all tested explants (cotyledons, hypocotyls, and roots) of all 3 tested cultivars. For other plant growth regulators, it was difficult to induce direct somatic embryogenesis (Table 1).

In this study, we tested the somatic embryogenesis capability of more than 20 additional Chinese and Australian commercialized cotton cultivars in order to develop an efficient protocol for cotton somatic embryogenesis and plant regeneration,. We obtained somatic embryogenesis from all of the tested cotton cultivars, although each yielded different somatic embryogenesis ratios (Table 2). According to (1) the somatic embryogenesis ratios, (2) the time required to induce somatic embryogenesis, and (3) the number of the somatic embryos produced in each culture, the tested cotton cultivars were divided into 3 groups: easily embryogenesis-induced cultivars, moderately easy embryogenesis-induced cultivars, and not easily embryogenesis-induced cultivars (Table 3).

For easily embryogenesis-induced cultivars, it was easy to induce somatic embryogenesis from a variety of explants on a wide range of medium using different combinations of plant growth regulators. It only took about 20-40 days to obtain embryogenic callus and/or somatic embryos at a high frequency from this group of cotton cultivars. Based on this study, easily embryogenesis-induced cultivars included CCRI 19, Simian No 3, CCRI 12, CCRI 13, Lumian 6, Jinmian 7, Jihe 321, Coker 201, Coker 312, and Sikral 1-3. For the group of not easily embryogenesis-induced cultivars, it generally took a much longer time (more than 100 days) to induce somatic embryogenesis callus and/or somatic embryos.

Fig 1. Cotton somatic embryogenesis and plant regeneration. a, non-embryogenic callus. b, non-embryogenic callus with embryogenic callus. c and d, direct somatic embryogenesis from cotton explants. e and f, high frequency somatic embryogenic callus with different developmental stage of somatic embryos. g, regenerated plantlet. h and i, regenerated plants transferred into soil.

For these types of cultivars, multiple subcultures on specific medium were required along with specific plant growth regulators in order to induce somatic embryogenesis. This group of cultivars included CCRI 17, CCRI 27, CCRI 30, and Yumian No 8. Although CCRI 27 produced somatic embryogenesis with a high frequency (8.0%) on MSB medium with 0.1 mg/L ZT, we classified CCRI 27 in the group of not easily embryogenesis-induced cultivars for two reasons. The first reason is that this cultivar took a long time to culture, usually more than 100 days. The second reason is that CCRI 27 did not easily produce somatic embryogenesis on other types of media except MSB medium with 0.1 mg/L ZT. Although it was hard to induce somatic embryogenesis from this group of cotton cultivars, we can still obtain plant regeneration from these not easily embryogenesis-induced cultivars after the screening of high embryogenic cell lines.

Table 3. Classification of cotton cultivars based on the capability of somatic embryogenesis

Type of embryogenesis-induced cultivars	Characteristics	Represented cultivars
Easy	Rapidly and easily produce embryogenic callus and somatic embryos with high frequency on many media.	CCRI 19, Simian No3, CCRI 12, CCRI 13, Lumian 6, Jinmian 7, Jihe 321, Coker 201, Coker 312, and Sikral 1-3
Moderately easy	After a long screening period, mature somatic embryos can be obtained; usually require 40-100 days to produce embryogenic callus	CCRI 16, CCRI 24, CCRI 29, Simian No 4, Lumian 1, and Jimian 12
Not easy	Embryogenic callus can be induced only on a certain medium with certain hormones; a long time is required for culture, usually more 100 days; and it is hard to obtain mature somatic embryos.	CCRI 17, CCRI 30, Yumian 8, and CCRI 27

Screening of highly efficient cell lines for somatic embryogenesis and plant regeneration

Although inducing cotton somatic embryogenesis on MSB medium with 0.1 mg/L ZT was relatively easy, this medium was not optimal for the growth and proliferation of embryogenic callus. Embryogenic callus should be transferred onto fresh MSB medium with 0.1 mg/L ZT and 2 g/L activated charcoal in order to promote growth and proliferation of embryogenic callus. Based on our study, 2 g/L of activated charcoal significantly enhanced the proliferation and differentiation of cotton embryogenic callus. After 21-28 days of subculture, the embryogenic callus with more extensive differentiation of somatic embryos was chosen and transferred onto fresh medium containing 0.1 mg/L ZT and 2 g/L activated charcoal. After screening several times, we obtained high frequency embryogenic cell lines for each of the 20 tested commercial cotton cultivars; each cell line could produce more than 100 somatic embryos in a short period of time (Fig. 1 e and f).

To test the effects of plant growth regulators or hormones on the proliferation of embryogenic callus and on the differentiation and development of cotton somatic embryos, 11 types of plant growth regulators were used. Cytokinins (ZT, KT, or 2iP) did not promote the proliferation of embryogenic callus (data not shown), but they did promote the differentiation and development of somatic embryos (Table 4). Embryogenic callus grown on MSB medium containing cytokinins (ZT, KT, or 2iP) produced more somatic embryos than embryogenic callus grown on MSB medium without phytohormones. We observed that a majority of these somatic embryos were mature embryos (torpedo-shaped embryos and cotyledon embryos). Out of the three cytokinins tested in this experiment, 2iP was considered the best for inducing the differentiation of cotton embryogenic callus. On MSB medium containing 0.1 mg/L 2iP, one gram of fresh embryogenic callus produced 339 somatic embryos, which is 3.85 times the amount of somatic embryos produced on MSB medium that did not contain any plant growth regulator or hormone. Auxins varied in their effects on somatic embryo differentiation and development; IAA and IBA slightly promoted the formation of somatic embryos. 2,4-D and TDZ functioned similarly as both inhibited the differentiation and development of cotton somatic embryos. The function of GA3 in cotton embryogenesis was similar to that of 2,4-D and TDZ. In contrast, ABA was shown to promote the formation of somatic embryos.

Mature embryos (cotyledon-shaped embryos and later torpedo-shaped embryos) were chosen and transferred onto fresh MSB medium containing 0.1 mg/L ZT and 2 g/L activated charcoal. More than 70% of mature embryos germinated into whole plantlets (Fig. 1g) after 15 to 25 days of culture. These regenerated plants were then successfully transferred into soil (Fig. 1 h and i). More than 90% of the plants showed normal morphology, were fertile, and produced seeds (Fig. 1i) while the rest did not produce any seeds or died during an early stage.

Discussion

Compared with other plant species, it is more difficult to induce somatic embryogenesis and plant regeneration in cotton. In the past 20 years, many laboratories have attempted to improve cotton tissue culture; *in vitro* cultured cotton cells have been induced to undergo plant regeneration via somatic embryogenesis using a variety of strategies (Aydin, et al. 2006; Divya, et al. 2008; Han, et al. 2009; Ikram ul 2005; Khan, et al. 2006; Kouakou, et al. 2007; Michel, et al. 2008; Mishra, et al. 2003; Shoemaker, et al. 1986; Trolinder and Chen 1989; Wang, et al. 2008; Zhang, et al. 2001; Zhang, et al. 2000). These regeneration procedures have been used to obtain genetically modified cotton after *Agrobacterium*-mediated transformation or particle bombardment

Table 4. Effect of phytohormones on differentiation and development of cotton somatic embryos

Phytohormone	Number of somatic embryos					% of control
	Globular-shaped embryo	Heart-shaped embryo	Torpedo-shaped embryo	Cotyledon embryo	Total	
GA	7	7	11	0	25 ± 1.7f	28.4
ABA	60	32	11	4	107 ± 5.2d	121.6
TDZ	26	0	0	0	26 ± 2.9f	29.5
2,4-D	20	4	0	0	24 ± 3.2f	27.3
IAA	77	39	10	10	136 ± 9.9d	154.5
IBA	63	34	4	4	105 ± 9.8d	119.3
BA	78	32	14	9	133±11.2d	151.1
KT	100	46	15	8	169±10.5c	192.0
ZT	144	58	28	8	238±11.8b	270.5
2iP	176	99	47	17	339±15.6a	385.2
BR	42	24	4	4	88 ± 5.5e	100.0
Control	46	30	8	4	88 ± 3.8e	100.0

Control means without any phytohormone. Values represent means of the number of somatic embryos per g of embryogenic callus obtained for each treatment. Means within a column followed by the same letters are not significantly different at $P<0.05$. All plant growth regulators were purchased from Beijing Biochemical Company.

(Cousins, et al. 1991; Ikram Ul 2004; Leelavathi, et al. 2004; Li, et al. 2009; Satyavathi, et al. 2002; Sunilkumar and Rathore 2001; Yuceer and Koc 2006; Zhang, et al. 2005). However, many obstacles still remain in cotton tissue culture. First, all of these procedures require multiple subcultures resulting in many somaclonal variations and long periods of tissue culture. Second, the plant regeneration frequency is very low. Finally and most importantly, all plant regeneration procedures are genotype-dependent. Cotton has more than 50 species, and each species has a different genotype. Until now, few cotton species have been induced to undergo plant regeneration via somatic embryogenesis and only a limited number of upland cotton cultivars have been induced to produce plant regeneration from somatic embryogenesis. The most responsive lines of cotton cultivars are Coker varieties, which are no longer being cultivated. Several attempts have been made to produce *in vitro* regeneration via somatic embryo-genesis for currently commercialized cotton cultivars but these attempts have been met with little success. Although Kumar and Pental (Kumar and Pental 1998), Zhang et al. (Zhang, et al. 2001; Zhang, et al. 2001), and several others (Han, et al. 2009; Khan, et al. 2006) have obtained regenerated plants from commercialized cultivars, the frequency of plant regeneration has been low and the regeneration procedure has required multiple subcultures that often produced unwanted somaclonal variation. Genotype-dependent plant regeneration response has also restricted the use of plant biotechnology for improving cotton breeding. Thus, transgenic cotton plants must be crossed with commercialized cotton cultivars in order to breed new commercialized cotton cultivars for cotton farmers.

The present report develops a simple procedure for cotton plant regeneration via somatic embryogenesis (Fig. 2). This protocol is based on a long term investigation that analyzes the effects of many inherited and environmental factors, such as phytohormones, genotypes, and the types of explants, on plant regeneration. This procedure can be employed to induce plant regeneration via somatic embryogenesis from a wide range of cotton cultivars. By adopting this procedure, the authors have obtained plant regeneration via somatic embryo-genesis from more than 20 Chinese and Australian commercialized cotton cultivars.

In previous reports, it was demonstrated that many plant growth regulators influence plant somatic embryogenesis and plant regeneration (Aasim, et al. 2009; Kendir, et al. 2009; Mamidala and Nanna 2009; Ozel, et al. 2006). Among these, 2,4-D was considered an essential plant growth regulator for the induction of somatic embryogenesis in cotton and in other plants (Elhag, et al. 2004; Michel, et al. 2008; Panaia, et al. 2004; Trolinder and Goodin 1987; Zhang, et al. 2000). In the early stages of cotton tissue culture, high concentrations of 2,4-D (usually 5-10 mg/L) are added into the medium; after obtaining callus, decreasing the concentration of 2,4-D promotes the initiation and formation of somatic embryos (Voo, et al. 1991). This procedure has been used in cotton tissue culture for many years. In this study, we found that ZT, a cytokinin, can induce somatic embryogenic callus from all of the tested cotton cultivars. The process of inducing somatic embryogenesis from plant cells is complicated.

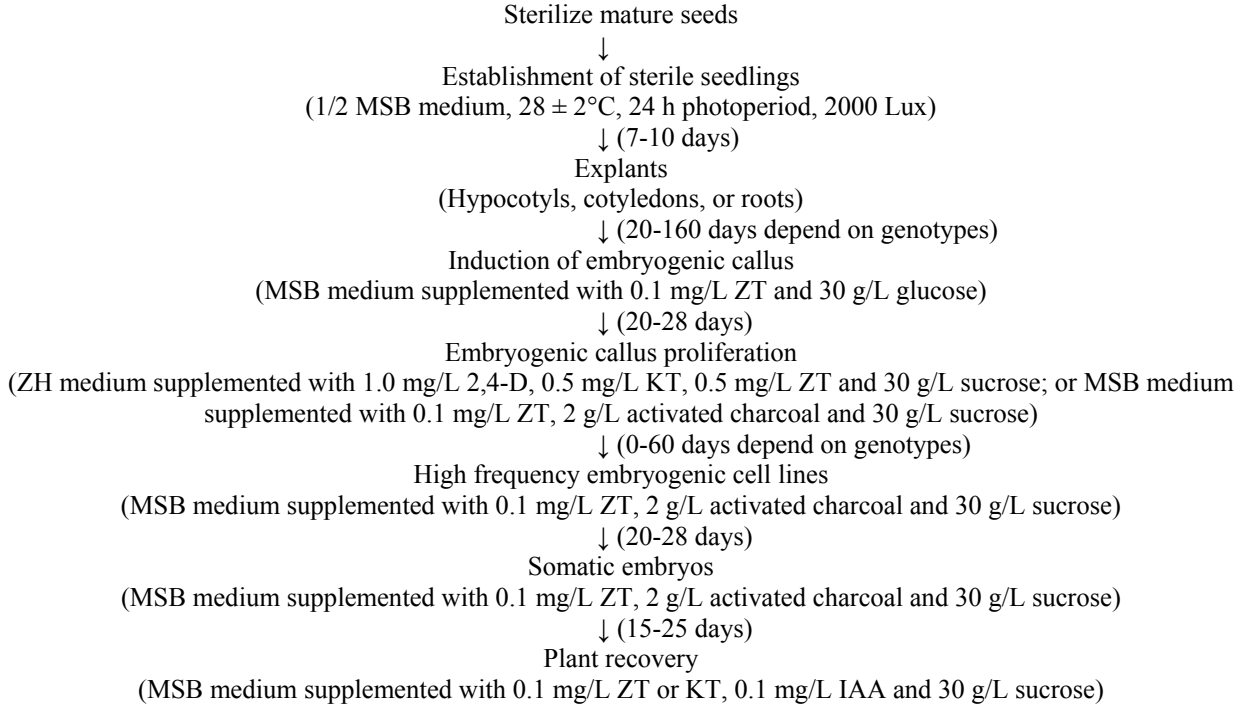

Fig 2. Protocol for somatic embryogenesis and plant regeneration of *Gossypium hirsutum* L.

Although several reports have shown that a variety of genes are differentially expressed during plant somatic embryogenesis, the regulatory mechanisms of these genes are unknown. A recently identified class of small RNAs, called microRNAs (miRNAs), may play an important role in plant somatic embryogenesis and plant regeneration. Increasing evidence has shown that miRNAs control almost all biological and metabolic processes in plants including developmental timing, organ differentiation and development, and response to environmental biotic and abiotic stresses (Zhang, et al. 2006). miRNAs have also been identified in cotton (Zhang, et al. 2007) and have been shown to be highly expressed in different cotton tissues and during different developmental stages (Zhang and Pan 2009). However, their regulatory mechanism in cotton somatic embryogenesis and plant regeneration needs to be further analyzed.

Another important factor that led to the success of this experiment was the application of desiccation stress in order to induce cotton somatic embryogenesis. In the early stages of cotton callus induction, two kinds of callus exist: embryogenic and non-embryogenic. In previous experiments, we found that non-embryogenic callus grew faster than embryogenic callus under optimal conditions that included rich nutrients and water (data not shown). However, embryogenic callus was more capable of survival than non-embryogenic callus (data not shown). Interestingly, under stressful conditions such as starvation or desiccation, non-embryogenic callus died first because of its low survival capability while embryogenic callus appeared after several cycles of growth. In an earlier investigation, Voo et al. (1991) studied the effects of physical and chemical desiccation on the germination of somatic embryos and plant regeneration. The highest frequency of plant recovery (40%) was observed when the embryos were subjected to physical desiccation. This result was reconfirmed by Chaudhary et al. (Chaudhary, et al. 2003). A recent study has also demonstrated that metabolic stress might enhance cotton somatic embryo maturation and eventually the conversion into plantlets (Kumria, et al. 2003). In this study, we found that dehydration and metabolic stress could help to induce somatic embryogenesis from a wide range of cotton cultivars; dehydration and metabolic stress also promoted the maturation of somatic embryos and plant recovery from somatic embryos.

Conclusion

This report establishes a simple procedure for highly efficient and rapid plant regeneration via somatic embryogenesis in cotton. The procedure has been

employed to obtain plant regeneration via somatic embryogenesis from more than 20 Chinese and Australian commercialized cotton cultivars, which account for more than 50% of the total cotton cultivated in China. This procedure promises to facilitate the application of plant genetic engineering on cotton genetic improvement.

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