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High efficiency organogenesis and analysis of genetic stability of the regenerants in *Solanum melongena*

Y. XING[1], Y. YU[1], X. LUO[1], J.-N. ZHANG[2], B. ZHAO[1] and Y.-D. GUO[1]*

College of Agriculture and Biotechnology, China Agricultural University, 100193 Beijing, P.R. China[1]
Institute of Vegetables, Shanxi Academy of Agricultural Sciences, 030031 Taiyuan, P.R. China[2]

Abstract

A novel protocol for plant regeneration from cotyledon explants of eggplant (*Solanum melongena*) reducing concentration of sucrose was established. The most efficient bud induction medium consisted of Murashige and Skoog (MS) medium supplemented with 2.0 mg dm^{-3} zeatin, 0.1 mg dm^{-3} indoleacetic acid and 10 g dm^{-3} sucrose. After 15 d, the shoot buds were fragmented and transferred to the shoot elongation MS supplemented with 1.0 - 2.0 mg dm^{-3} gibberellic acid and 4.0 - 8.0 mg dm^{-3} $AgNO_3$, which promoted shoots elongation. The genetic stability of the regenerated plants was analyzed by flow cytometry, RAPD and SSR molecular markers. The results indicated that almost no somaclonal variation was detected among the regenerants.

Additional key words: $AgNO_3$, eggplant, flow cytometry analysis, RAPD, SSR.

Introduction

Eggplant (*Solanum melongena* L.), an important vegetable crop worldwide, is highly susceptible to several diseases and pests that cause serious crop losses. One of the solutions to this problem is the development of resistant eggplant cultivars using genetic transformation techniques, which in turn require efficient and stable plant regeneration protocols. Eggplant has been regenerated *via in vitro* organogenesis from different cultured explants such as stem, hypocotyl, leaf, cotyledon and root (Kamat and Rao 1978, Mukherjee *et al.* 1991, Sharma and Rajam 1995, Magioli *et al.* 1998, Franklin *et al.* 2004). Although eggplant tissue presents a high morphogenetic potential that is useful for further studies to develop genetic transformation, the progress of plant regeneration in eggplant is relatively limited due to the formation of ill-formed buds and the callus which resist elongation (Kamat and Rao 1978, Mukherjee *et al*. 1991). Moreover, although high frequency of bud formation were achieved in the recent studies, only limited buds could elongate and grow normally (Franklin *et al.* 2004). Magioli *et al.* (1998) found that the leaves and cotyledons were the most responsive explants and the shoot bud induction rate was high by using thidiazuron, however, the shoots were short and failed to develop roots. Sanatombi and Sharma (2008) indicated that the medium containing indoleacetic acid was the best medium for elongation of the shoot buds of *Capsicum*, Sreedhar *et al*. (2008) reported that the dosage of sucrose effected significantly the shoot elongation of *Stevia rebaudiana*.

In plant regeneration, one of the most crucial concerns is to retain genetic integrity with respect to the mother plants. It is known that *in vitro* culture techniques could induce genetic variability (Larkin and Scowcroft 1981). Some strategies are available for detecting genetic variation, including flow cytometry analysis and DNA analysis techniques. Flow cytometry was performed to study DNA content stability of regenerated plants which offers the possibility of fast and large scale analysis of the DNA content of cells for a genotype of purposes, *e.g.* determination of species specific DNA amount, analysis of the cell cycle activity in different tissues and measurement of endopolyploidization levels (Nassour *et al.* 2003, Guo *et al*. 2005, Borchert *et al.* 2007). Somaclonal variability can also be evaluated by DNA analysis techniques, such as random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR/ISSR). The RAPD markers

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Abbreviations: GA_3 - gibberellic acid; IAA - indoleacetic acid; MS medium - Murashige and Skoog medium; RAPD - random amplified polymorphic DNA; SSR - simple sequence repeat; ZT - zeatin.
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* Corresponding author. fax: (+86) 10 6273 4389, e-mail: yaguo@cau.edu.cn

are extensively used to assess genetic variation generated by *in vitro* techniques (Qin *et al.* 2006, 2007, Hussain *et al.* 2008), while SSR/ISSR are efficient molecular markers that have been mainly used to measure genetic similarity or dissimilarity (Becher *et al*. 2000, Yao *et al.* 2008, Chandrika *et al.* 2008). These markers offer the advantage of being simpler to use, less plant material to require, less expensive and less time-consuming than other DNA markers such as restriction fragment length polymorphism (RFLP) and amplification fragment length polymorphism (AFLP), which can be conveniently used to rapidly evaluate somaclonal variability in regenerated plants.

The present study describes an efficient system for the plant regeneration from cotyledons of eggplant and was designed to detect the genetic variation for regenerated plants by flow cytometry analysis, RAPD and SSR. To our knowledge, this is the first report on detecting genetic variation in regenerated plants of eggplant, and retaining genetic integrity would be useful for transformation and breeding programs.

Materials and methods

Four *Solanum melongena* L. genotypes (Meizi, Xianfeng I, Heijuren and Jiuye) were used in the current study. The seeds were presoaked for 8 - 10 h in sterile distilled water, then were surface-sterilized with 2 % NaClO solution for 20 min and rinsed with sterile distilled water. The seeds were sown in culture tubes containing Murashige and Skoog (1962; MS) medium with 30 g dm^{-3} sucrose and 8 g dm^{-3} agar, and adjusted to pH 5.8 before autoclaving at 121 °C for 20 min. Incubation was in a growth chamber at temperature of 26 ± 2 °C and 14-h photoperiod (irradiance of 40 μmol m^{-2} s^{-1} provided by fluorescent tubes). Cotyledons were excised aseptically from 10-d-old seedlings.

The cotyledons were cut into halves and thereafter used as explants. Nine explants were inoculated in a Petri dish (90 mm in diameter). The shoot induction medium consisted of MS medium supplemented with zeatin (ZT) at 1.0 - 6.0 mg dm^{-3}, indoleacetic acid (IAA) at 0.1 - 0.5 mg dm^{-3} in combination with sucrose at 2.5 - 30 g dm^{-3} and agar at 8 g dm^{-3}. The explants were incubated in a growth chamber under the same conditions as mentioned above. After a 15-d incubation, the shoots were excised from the cotyledons and placed on shoot elongation medium containing MS medium supplemented with ZT at 1.0 - 6.0 mg dm^{-3}, IAA at 0.1 - 0.5 mg dm^{-3} in combination with gibberellic acid (GA_3) at 0 - 4.0 mg dm^{-3} and silver nitrate ($AgNO_3$) at 0 - 12.0 mg dm^{-3}, sucrose at 2.5 - 30 g dm^{-3} and agar at 8 g dm^{-3}, pH 5.8. The GA_3 and $AgNO_3$ were filter-sterilized and added after the autoclaving. The cultures were incubated for four weeks under the same conditions as above.

The elongated shoots (2 cm in length) were excised from the explants and inoculated in glass tubes containing half-strength MS medium for rooting. The cultures were incubated under the same conditions as above. After rooting, the plantlets were transferred to pots containing peat and *Perlite* (2:1) and placed in a greenhouse under plastic covers to maintain a high humidity.

Flow cytometry was performed to study DNA content stability of regenerated plants. The young leaves were chopped with a razor blade in 2 cm^3 nucleus isolation buffer including propidium iodide for fluorescent DNA staining (Marie and Brown 1993). After filtration through a 50 μm nylon sieve, the DNA content of the isolated nuclei samples were analyzed by a flow cytometer (*FACSCalibur*, San Diego, USA). Fluorescence of eggplant nuclei was compared with an internal reference of *Lycopersicon esculentum* cv. Stupické nuclei (2C = 1.95 pg, Borchert *et al*. 2007) after logarithmic amplification. The absolute DNA amounts of the samples of eggplant were calculated based on the values of the G_1 peak of eggplant and tomato.

The RAPD analysis was used to study genetic stability of regenerated plants of eggplant. The total DNA was extracted from 200 mg young leaf tissue by the cetyl-trimethylammonium bromide (CTAB) method (Guo *et al*. 2003). A total of 42 random 10-mer primers (*Sangon*, Shanghai, China) were used for RAPD analysis, and 16 of them were listed in Table 1. The PCR was carried out in 0.02 cm^3 volume containing 20 - 30 ng genomic DNA, 0.002 cm^3 of 10× buffer (1.5 mM $MgCl_2$), 200 μM dNTPs, 1 μM primer and 1.0 unit Taq polymerase (*Takara Shuzo Co*., Shiga, Japan). The PCR amplification was performed on a thermal cycler (*TC-312*, *Techne*, Duxforg, UK) programmed for 5 min at 94 °C, followed by 38 cycles of 45 s at 93 °C, 30 s at 36 °C, 80 s at 72 °C and a final stage of 6 min at 72 °C. The RAPD amplification products were separated by electrophoresis on a 1.3 % (m/v) agarose gel in 0.5× TBE. The molecular mass ladder DNA marker III (*Tiangen*, Beijing, China) were used for band sizing.

Table 1. Primers used in RAPD analysis of genetic stability in eggplant regenerants.

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
C-1	CAGAAGCGGA	C-9	GGGTAACGCC
C-2	TGAGCCTCAC	C-10	CCAGATGCAC
C-3	ACGATGAGCC	C-11	ACGCAGGCAC
C-4	TGGCCCTCAC	C-12	ACGATGAGCC
C-5	CATTCGAGCC	C-13	TGCGGCTGAG
C-6	GTGTCGCGAG	C-14	ACGGCGTATG
C-7	TCACGTCCAC	C-15	CTACGCTCAC
C-8	AGGGCCGTCT	C-16	ACCTGGACAC

SSR analysis of nuclear genomes using 13 primer pairs (Table 2) described by Nunome *et al*. (2003) and Behera *et al*. (2006) were conducted. The reaction mixture (0.01 cm^3) contained 10 - 20 ng genomic DNA, 0.001 cm^3

of 10× buffer (1.5 mM $MgCl_2$), 200 μM dNTPs, 0.5 μM primer (each of forward and reverse primer) and 0.5 U Taq polymerase (*Takara Shuzo Co.*). The PCR cycles were programmed as follows: one initial denaturing cycle at 94 °C for 4 min, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 50 s at 72 °C and a final cycle of 6 min at 72 °C. The molecular mass ladder 100 bp DNA marker (*Tiangen*) were used for band sizing. The amplified products were electrophoresed on 8 % polyacrylamide gels. After electrophoresis, the gel was stained with silver nitrate and photographed using a digital camera.

Table 2. SSR primer pairs used to verify the nuclear genome stability in regenerants of eggplant.

EM No.	Repeat motif	Forward primer sequence (5'-3')	Backward primer sequence (5'-3')	Annealing temperature [°C]
104	(TC)9(AC)38(AT)19	TGGATCTGCAAAGAAAAGGAGAAAG	CGCAAATCGGGTAGACTTTCGAT	58
107	(AC)13(AT)7	GGCCCTAGACTGAGCTGAAATGTT	TGCTACAACCAACACAACCCTCAA	61
114	(AC)13	AGCCTAAACTTGGTTGGTTTTTGC	GAAGCTTTAAGAGCCTTCTATGCAG	60
116	(AC)12(AT)8	TTAGAAATTTCGGAACAAAGAGA	CCACATGAAACTTGGACCAATGAG	62
126	(AT)7(GT)18	GCATAGCTTATGAGTCAGGTGGCTTT	GCTCATCAAACCATCACATTCAAG	61
127	(AC)13(AT)13	CAGACACAACTGCTGAGCCAAAAT	CGGTTTAATCATAGCGGTGACCTT	60
128	(CA)26(TA)19	TAGCGGTGCTAGGTCATCATCTCA	TTCTCAAGAAGTTGCTCCAAAGGA	62
134	(GT)2GC(GT)6	AGTAAGGGAAAGTGCTGACGAAGG	CAGAGTCATCGTTATGGGGAGGTT	62
135	(CA)11(GA)20	ATCCTGTTGCTGCTCATTTTCCTC	AGGAGGATCCAAGAGGTTTGTTGA	61
139	(AC)6AT(AC)11(AT)10	TGCTAAGTCGTCATCCCACAAGAA	GATTTTGGCTCCTTGACCATTTTG	61
141	(AT)16(GT)19	TCTGCATCGAATGTCTACACCAAA	AAAAGCGCTTGCACTACACTGAAT	60
151	(TG)3TA(TG)8(TA)6	TGATTTGGCCCTTAAGCCTAAGTATG	GACTCCTCAAGCCTTTACCTCCAA	62
155	(CT)38	CAAAAGATAAAAAGCTGCCGGATG	CATGCGTGAGTTTTGGAGAGAGAG	62

Results and discussion

In our study, the response of cotyledon explants was 100 %. The highest frequency of shoot formation was obtained on medium containing 2.0 mg dm^{-3} ZT combined with 0.1 mg dm^{-3} IAA (28.1 shoots $explant^{-1}$). The number of shoots increased with the time of incubation and the maximum number was obtained in 13-d cultures (Fig. 1*A*, Table 3). The mean number of shoot per explant decreased sharply in the further culture due to over growth of callus in the region of shoot formation (data not shown), this result was consistent with the former studies (Mukherjee *et al*. 1991, Kamat and Rao 1978, Picoli *et al*. 2001).

Table 3. Effect of growth regulator combinations on bud differentiation of eggplant Heijuren in 13-d culture (sucrose 30 g dm^{-3}). Means ± SD, $n \geq 5$. Means followed by the same letters are not significantly different according to the LSD test at the 5 % level of probability.

ZT [mg dm^{-3}]	IAA [mg dm^{-3}]	Explant with shoots [%]	Shoot number [$explant^{-1}$]
1.0	0.1	100	15.0 ± 5.6bc
	0.5	95	7.7 ± 2.9a
2.0	0.1	100	28.1 ± 7.0e
	0.5	100	22.6 ± 6.7d
4.0	0.1	100	13.6 ± 6.1b
	0.5	100	17.3 ± 7.1c
6.0	0.1	92	7.9 ± 2.9a
	0.5	100	9.0 ± 2.8a

The concentration of sucrose played an important role on promoting shoot differentiation and inhibiting callus formation from explants. When the explants of Heijuren were cultured on the medium containing 5 - 10 g dm^{-3} sucrose, the shoots could grow normally, and the formation of callus could be inhibited (Fig. 1*B*). The highest shoot differentiation (20.8 shoots per explant) was obtained on the medium containing 10 g dm^{-3} sucrose. The similar results were observed in other three genotypes (data not shown). The results indicated that the concentration of sucrose in the culture medium had an important influence on shoot organogenesis, and this was in agreement with the results achieved in the previous study on eggplant (Mukherjee *et al*. 1991).

The average shoot number per explant varied greatly among the genotypes. The Heijuren achieved the best result in shoot regeneration capacity, whereas genotype Meizi showed the lowest capacity of shoot formation (Table 4). The results indicated that the genotype played an important role in the shoot formation which was in line with a former study (Sharma and Rajam 1995).

The shoot buds from the cotyledon explants did not elongate normally. The similar results were reported previously. Magioli *et al* (1998) found that prolonged culture of morphogenesis calluses on medium containing TDZ often resulted in the formation of short, vitrified shoots which would be not easy to elongate and develop

roots normally. Franklin *et al*. (2004) obtained a large number of buds from a single explant (root), however, the number of elongated shoots was small.

Table. 4. Shoot induction from explants of four genotypes on the bud induction medium (SI medium: MS supplemented with 2.0 mg dm^{-3} ZT, 0.1 mg dm^{-3} IAA, 1 % sucrose, 0.8 % agar) and the shoot buds elongation medium (SI medium supplemented with 8.0 mg dm^{-3} $AgNO_3$, 1.0 mg dm^{-3} GA_3). Means followed by the same letters are not significantly different according to the LSD test at the 5 % probability level. Means ± SD, $n \geq 5$.

Genotypes	Bud number [explant^{-1}]	Shoot elongation [%]
Meizi	9.0 ± 3.3c	70.0
Xianfeng I	15.6 ± 5.5b	46.7
Heijuren	24.5 ± 6.2a	76.7
Jiuye	17.6 ± 5.3b	53.3

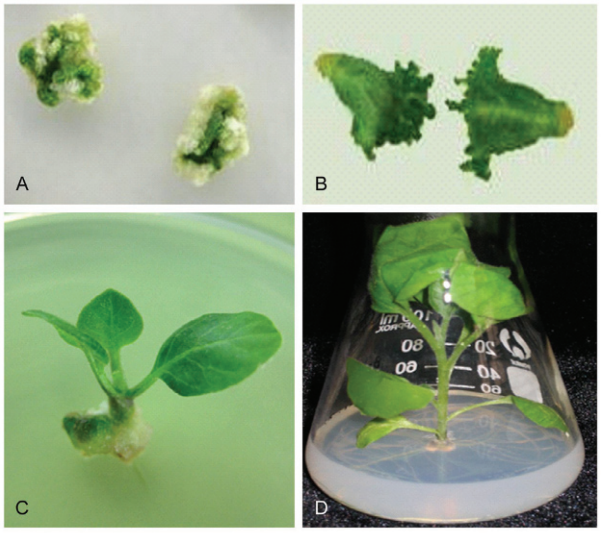

Fig. 1. Shoot differentiation from cotyledon explants and plant regeneration in eggplant cv. Heijuren: *A* - the number of shoot buds decreased sharply due to over growth of callus on the differentiation medium containing 30 g dm^{-3} sucrose after 20 d; *B* - shoot buds formation in the medium containing 10 g dm^{-3} sucrose after 20 d; *C* - an elongated shoot from cotyledon explants; *D* - a regenerated plant with roots.

$AgNO_3$ and GA_3 were proved to be essential in shoot buds formation and elongation in *in vitro* plant regeneration (Hyde and Phillips 1996, Mohinuddin *et al*. 1997). In general, GA_3 is in favor of shoot stem elongation, and silver has been associated with ethylene action and is widely used as inhibitor of ethylene perception (Ciardi and Klee 2001). The promotory effect of silver nitrate has been reported in cucumber (Mohinuddin *et al*. 1997) and cassava (Zhang *et al*. 2001).

The presence of $AgNO_3$ and GA_3 showed a remarkable effect on shoot elongation in the cotyledon cultures of eggplant (Fig. 1*C*). The response varied with different concentrations of $AgNO_3$ and GA_3 used. The medium fortified with $AgNO_3$ at 4.0 - 12.0 mg dm^{-3} and GA_3 at 1.0 - 2.0 mg dm^{-3} was found suitable for increased shoot bud induction. The highest rate of shoot formation and elongation for genotype Heijuren was induced by the combination of 8.0 mg dm^{-3} $AgNO_3$ and 1.0 mg dm^{-3} GA_3 (Table 5). The similar results were obtained in other three genotypes (data not shown). The normal elongated shoots were transferred to half-strength MS medium for rooting (Fig. 1*D*). After 20-d culture, the healthy and vigorous roots were formed, the regenerated plantlets were then transplanted *ex vitro*.

Table 5. Effect of various concentrations of GA_3 and $AgNO_3$ on shoot elongation from cotyledons of Heijuren on MS medium with 2.0 mg dm^{-3} ZT and 0.1 mg dm^{-3} IAA. Means followed by the same letters are not significantly different according to the LSD test at the 5 % probability level.

GA_3 [mg dm^{-3}]	$AgNO_3$ [mg dm^{-3}]	Number of shoots > 1.5 cm	Shoot elongation [%]
0.0	0.0	2	6.7d
1.0	4.5	17	56.7bc
1.0	8.1	23	76.7a
1.0	12.0	15	50.0c
2.0	4.0	14	46.7c
2.0	8.0	20	66.7ab
2.0	12.0	13	43.3c

Nuclear DNA content (genome size) is very useful for systematic purposes and evolutionary considerations (Bennett and Leitch 1995). Angiosperm DNA C-values are highly variable, differing over 1000-fold. Genome size is positively correlated to nuclear volume, cell volume, mitotic cycle time and the duration of meiosis. Flow cytometry has been demonstrated to be a convenient and rapid method for estimating the nuclear genome size of plants. Altered DNA contents are common mutations reported as a consequence of *in vitro* regeneration (Kubaláková *et al*. 1996, Guo *et al*. 2005). Tissue culture conditions are considered as a cause for chromosome instabilities although the mechanism is still unknown (Phillips *et al*. 1994, Guo *et al*. 2005).

In order to test the genetic variability of the regenerated plants, leaves of eggplants were subjected to flow cytometric analysis with tomato leaves as an internal standard and the absolute DNA contents of 40 regenerated plants were calculated. The nuclear DNA amounts of the used standards were determined based on the value of 1.95/2C pg for *Lycopersicon esculentum* cv. Stupické (Borchert *et al*. 2007). Here, highly constant values were obtained, the nuclear DNA content of seedlings was 2.43 pg whereas the nuclear DNA contents of 40 regenerated plants were 2.43 or 2.42 pg, which showed no significant differences (*ANOVA*, Tukey-HSD) among the regenerated plants and seedlings.

DNA markers are attractive methods for examining somaclonal variation. The RAPD and SSR techniques have been applied to investigate genetic variability and

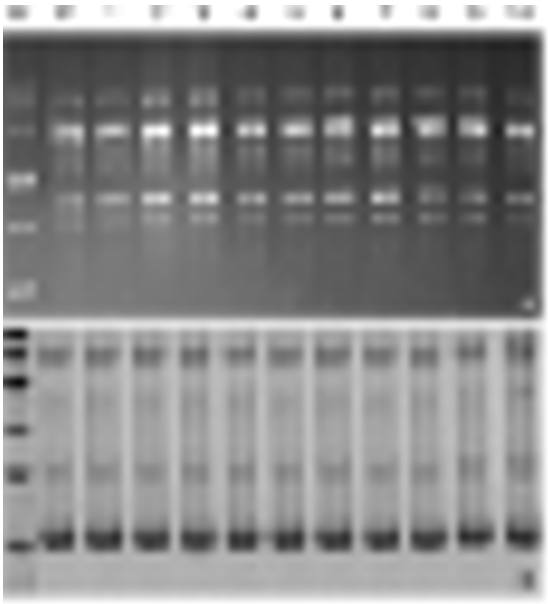

Fig. 2. Analysis of genetic stability of the regenerated plants by RAPD and SSR markers. *A* - RAPD profile of plantlets regenerated from eggplant obtained with the primer C-6. *Lanes* 1 - 10: regenerated plantlets, *lane P*: mother eggplant; M is DNA size marker, bands from the bottom: 500, 800, 1200, 2000, 3000 and 4500 bp. *B* - SSR analysis with the primer EM-114. *Lanes* 1 - 10: regenerated plantlets, *lane P*: mother eggplant; M is DNA size marker, bands from bottom: 200, 300, 400, 500, 600 and 700 bp.

were considered to be efficient and reliable (Dayanandan *et al*. 1998, Rahman *et al*. 2000, Yang *et al*. 2008).

For RAPD analysis, we have screened a total number of 42 arbitrary 10-mer primers, and 40 random-selected regenerated plants were employed in the RAPD amplification. Of these 42 primers, 16 gave clearly identifiable bands and these were therefore used further in PCRs (Table 1). The 16 primers used in this analysis yielded 91 scoreable bands with an average of 5.7 bands per primer. Fig. 2A showed the RAPD amplification using primer C-6 in genotype Heijuren, the results demonstrated the genetic stability of regenerants. In the present test, no genetic variation of the regenerated plants of eggplant was detected by RAPD.

For SSR analysis, 13 SSR DNA loci were used to examine somaclonal variation (Table 2) for 40 plants selected randomly among the regenerated plants. In the present study, no SSR DNA variation was observed among the somaclones at all SSR loci. Fig. 2*B* depicted a representative sample of the microsatellite DNA profiles of the sampled plantlets of eggplant at SSR loci (EM-114), showing no somaclonal variation in the genotype of Heijuren. We were not able to detect any somaclonal variation among the regenerated plants from the same genotype by the SSR molecular analysis in the current study.

The current results demonstrated no genetic variation of the regenerated eggplants. A proposed explanation is that the plants regenerated from unorganized callus vary more than those from organized callus, whereas no or very little variation occurs when plants are regenerated directly without an intermediate callus phase (Leroy *et al*. 2001, Sliwinska and Thiem 2007). It may be the reason that a fast and efficient organogenesis will be beneficial to a steady protocol of eggplant and the process of transformation.

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