Optimization of *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.) immature embryos

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Abstract

Sunflower is an important oilseed crop, however the genetic transformation of sunflower has been very challenging to date. In the present study, we describe a successful Agrobacterium-mediated transformation approach via somatic embryogenesis from immature zygotic embryos of sunflower. By optimizing the culture medium, we have achieved high efficiency (45.5%) of somatic embryogenesis with MS medium containing 0.4 mg/l 2,4-D and 120 g/l sucrose when embryo size was less than 2mm. *Agrobacterium tumefaciens* LBA4404 harboring pGBI121-LycB was transformed into 1500 immature sunflower embryos derived from 4 different genotypes. Four strains of transgenic plants were obtain through kanamycin screening and subsequent validation via PCR and Southern Blot confirmed 3 positive strains, in which the transgenes were integrated into the genome of sunflower.

Keywords: Agrobacterium; genetic transformation; immature embryos; sunflower.
Abbreviations: 2,4-D-2,4-Dichlorophenoxyacetic acid; MS-Murashige & Skoog's medium.

Introduction

The cultivated sunflower (Helianthus annuus L.) is an annual plant with high nutrition value. The oilseed sunflower has been one of the most important oil-producing crops all over the world. Tissue culture technology of sunflower has been under investigation since the 1980s, but regeneration of this crop is still limited to date. Regeneration of sunflower can be achieved through organogenesis (Pugliesi et al., 1991; Sarrafi et al., 1996) or embryogenesis (Zezul et al., 1995; Fambrini et al.,1997; Laparra et al.,1997). The regeneration frequency of sunflower is affected by culture conditions, plant hormones and genotypes, as well as their reciprocity. Prado et al. (1990) reported that the somatic embryo from hypocotyls of sunflower could only be induced in liquid medium as this condition enhanced the contact between the tissue and the medium. Although embryogenesis increased with increasing sucrose concentration, organogenesis showed the opposite trend (Geneviève, et al., 1995). In addition, darkness played an important role in achieving a high efficiency of somatic embryogenesis (Fiore, et al., 1997). Here we use immature embryos derived from 4 different sunflower genotypes as explants to induce somatic embryogenesis. The genotype with the highest efficiency of somatic embryogenesis was chosen for *Agrobacterium* transformation. This successful transformation method provides a theoretical and technical basis for sunflower genetic engineering, the generation of transgenic seed and the selection of mutants.

Results

The regions of somatic embryogenesis

Our tissue culture results showed that some immature embryos could develop into white watery callus, however only calluses that developed green spots could differentiate into new organs. Some immature embryos were able to develop directly to somatic embryos or to both small calluses and embryos. No critically active regions determining the development of embryos were identified. Embryos could be either induced from callus (Fig. 2D), or the middle part of two cotyledons (Fig. 2B), or the marginal area of the cotyledon (Fig. 2C and E), or half cotyledon (Fig. 2A and F).

Effect of medium compositions on somatic embryogenesis

Among plant hormones tested in various combinations and concentrations, the medium supplemented with 2, 4-D significantly stimulated the induction of somatic embryos. The combination of 120 g/l sucrose and 0.4 mg/l 2, 4-D led to a maximal frequency of embryogenesis (Table 1). Three different carbohydrates were tested in three different concentrations and the highest frequency of somatic embryogenesis was achieved in medium containing sucrose, followed by glucose，while medium with maltose was found to inhibit the somatic embryogenesis of sunflower (Fig. 3A). A series of concentration of sucrose (40~240 g/l) in medium with 0.4 mg/l of 2, 4-D was selected for further studies. The results indicated that the highest frequency of somatic embryogenesis was observed with medium containing 120 g/l sucrose, whereas embryos failed to develop on medium with 40 g/l sucrose (Fig. 3B), suggesting high concentrations of sucrose (12%) would change the osmolarity of medium and increase cytosolic concentration of somatic embryos, which could affect the development of somatic embryos directly.

Effects of genotype and immature embryo size on embryogenesis

The effect of genotype on the formation of shoot regenerating and callus from zygotic embryos was reported (Espinasse et
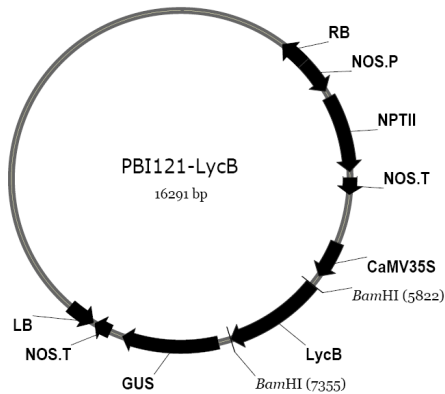
Fig 1. Schematic diagram of plasmid pBI121-LycB.

Fig 2. Development of somatic embryos and regeneration of sunflower cultured on MS medium containing 0.4 mg/l 2, 4-D and 120 g/l sucrose. (A) and (F) Somatic embryos on half cotyledons. (B) Somatic embryos on the middle of the two cotyledons. (C) and (E) Somatic embryos along the margins of the cotyledon. (D) Callusing and somatic embryo differentiation. (G) and (H) *Agrobacterium* transformed plantlets.

al., 1989). In our studies, RHA266 presented the highest frequency of somatic embryogenesis (38.4%) and 2736 was the lowest (10.1%) (Fig. 3C), indicating some genotypes required specific conditions. The effect of immature embryo size of sunflower on shoot regeneration was shown in previous studies (Wetherell, 1984; Finer, 1987). The frequency of embryogenesis decreased as embryo size increased, on MS medium with 0.4 mg/l 2, 4-D and 120 g/l sucrose, and the larger embryos were more ofen develop into watery callus (Fig. 3D). The highest frequency (45.5%) of somatic embryogenesis was obtained from immature embryos shorter than 2 mm, and the corresponding callus was small (Fig. 3D).

Agrobacterium-mediated transformation of immature embryo

Four sunflower genotypes including RHA266, PR29, HA300 and 2603, were transformed with *Agrobacterium tumefaciens* LBA4404 haboring plasmid pBI121-LycB. The transgenic plants were identified through kanamycin screening. As Table2 shows, we finally collected 2 lines of kanamycin-resistant transgenic plants, and transformation efficiency reached 1% as immature embryos of HA300 were inoculated for 5 min in Agrobacterium culture (OD_{600}=0.4) and co-cultivated for two days. Different genotypes of sunflower had different transformation efficiency. Transformation efficiency of 1% and 0.33% were obtained for the genotypes HA300 and RHA266, respectively, under 0.4 (or 0.5) OD value of bacterium cells, 5-minute inoculation and two-day co-cultivation. Four out of 1500 sunflower immature embryos from 4 genotypes survived kanamycin screening in total，and 3 of them passed PCR validation (Fig. 4A). Southern blot results showed two T-DNA fragments (3027bp and 1280bp) of pBI121-LycB had been identified from three individual lines, further indicating transgenes were successfully integrated into the sunflower genome (Fig. 4 B).

Discussion

It is believed that somatic embryogenesis can be produced through tissue culture in 40 families for more than 100 species (Hu, 1982). But the capacity of plants to produce somatic embryos is different for different families and genuses. Even among plants from the same genus, the efficiency of somatic embryogenesis differs significantly. In our study, under identical conditions, including the same size of immature embryo and culture conditions, the transformation frequency varied significantly between genotypes, with the highest up to 38.4% and the lowest at only 10.1%. Our data support the proposition that the difference could be attributed to two factors: different genetic backgrounds and different optimal culture conditions required for different genotypes (Cui et al., 1993). The influence of hormones on the induction of embryogenesis is very important in plant tissue culture. One of the critical hormones is phytohormone 2, 4-D, which has been shown to play an important role in the creation of early somatic embryos in maize and carrots (Sujatha and Prabakaran, 2001; Bronsema, 2001). Our results confirmed 2,4-D importance in sorghum somatic embryogenesis. A possible explanation is that endogenous hormones could be regulated by 2, 4-D, reaching a new equilibrium in the plant cells. Then, upon the cells beginning to divide, the process of embryogenesis is triggered, and the early development of somatic embryos was induced (Cui and Dai, 2000). *Agrobacterium tumefaciens*-mediated genetic transformation has proved a powerful method for gene transfer among species. It is generally used for genetic transformation of higher plants and offers many advantages including the integrity of foreign DNA, high stability of the inserted fragment, the capacity for larger fragments and low frequency of mutation (Gelvin, 2003). By August 2010, 27 articles have reported successful sunflower transformation through *Agrobacterium tumefaciens*. Explants used in these papers were cotyledons, stem apexes, hypocotyls or immature hypocotyls rather than immature embryos (Pugliesi et al., 1993; Manavella and Chan, 2009; Schrammeijer et al., 1990; Gürel and Kazan, 1999; Hartman, 1991; Dagustu et al., 2008). In our studies, the immature embryos from 4 genotypes of sunflower were

Table 1. Effect of sucrose and 2, 4-D on the frequency of somatic embryogenesis of sunflower.

Treatment		Frequency of embryogenesis (%)	Average frequency of embryogenesis at different sucrose concentrations (%)
Sucrose (g/l)	2,4-D (mg/l)		
40	0.4	1	
40	0.8	0	0.33c
40	1.2	0	
80	0.4	21.5	
80	0.8	12.5	17.53b
80	1.2	18.6	
120	0.4	42.1	
120	0.8	32.8	29.47a
120	1.2	21.7	
160	0.4	34.2	
160	0.8	29.8	32.20a
160	1.2	24.4	

Means followed by different letters are significantly different (p<0.05).

Table 2. The *Agrobacterium*-mediated transformation efficiency of different genotypes of immature embryos of sunflower.

Genotype	COA* (OD$_{600}$)	TOI* (min)	TOCC* (day)	NOE*	NOPRK*	NOPPP*	FOT* (%)
	0.4	10	2	200	0	0	0
HA300	0.4	10	3	200	0	0	0
	0.4	5	2	200	2	2	1
RHA266	0.5	5	2	300	2	1	0.33
PR29	0.6	4	3	300	0	0	0
2603	0.8	5	3	300	0	0	0

*COA：Concentration of *Agrobacterium* cells; TOI: time of inoculation; TOCC: time of co-cultivation; NOE: no. of explants; NOPRK: no. of plantlets resistant kanamycin; NOPPP: no.of PCR-positive plantlets; FOT: frequency of transformation.

cultured as explants, and 0.2% transformation efficiency was obtained, confirmed by antibiotic-resistance screening, PCR and southern blot. Among 4 genotypes of sunflower, HA300 achieved up to 1% transformation efficiency and proved a high transformation efficiency genotype in accordance with previous reports (Escandón and Hahne, 1991; Müller et al., 2001; Molinier et al.*,* 2002; Weber et al., 2003). Intriguingly, no young transgenic plants were identified from PR29 and 2603. Our results further indicated interaction between the genotypes and *Agrobacterium tumefaciens* strains influenced the transformation efficiency. In addition, infection time, bacterium concentration and co-culture days also had significant effect on the transformation efficiency.

Materials and methods

Plant material

Five sunflower genotypes were used, RHA266, HA300, 2603, 2736 and PR29, supplied by The Research Center of Plant Genetic Engineering, Tianjin University, China.

Induction of Somatic Embryogenesis

Achenes of sunflower with immature embryos were collected 5-10 days after pollination, and washed under running tap water for 30 min. Materials were then sterilized with 75% alcohol for 5-6 min on the surface and rinsed four times, for 5 min, in sterile distilled water. Explants (immature zygotic embryos) were removed from the achenes using tweezers and scalpels and cultured on media for embryogenic tissue induction. The achenes were categorized in terms of embryo size: a (≤2 mm), b (2-4 mm), c (4-6 mm), d (6-8 mm) and e

(≥8 mm). Nutrient medium consisted of major and minor salts as well as vitamins according to Murashige et al. (1962), sugar source, 0.7% agar (Sigma) and supplemented with or without 2,4-D. The pH of each medium was adjusted to 5.8 before addition of agar. To test the effect of sugar source and concentration, explants were incubated on the basal medium, but supplemented with 3 different concentrations (40, 80, 120 g/l) of sucrose, glucose or maltose.

Plasmid DNA construction and Agrobacterium strains

The plasmid pBI121-LycB was constructed for transformation of *Agrobaterium tumefaciens* LBA4404. It contains the neomycin phosphotransferase-II (NPT II) gene responsible for resistance to the antibiotic kanamycin sulphate, and the Lycium barbarum lycopene beta-cyclase (LycB) gene（Genbank accession number: AY920918; Fig. 1).

Plant transformation

LBA4404 harboring the plasmid pBI121-LycB were cultured to OD$_{600}$ =0.6 at 28℃ and 170 rpm in YEB medium containing the appropriate antibiotics, harvested by centrifugation and resuspended in iso-volume liquid MS medium. The immature embryos of sunflower were incubated for 20 min, then transferred to 50 ml glass containers with solidified MS medium and co-cultivated for 48 h at 25℃ in the dark.
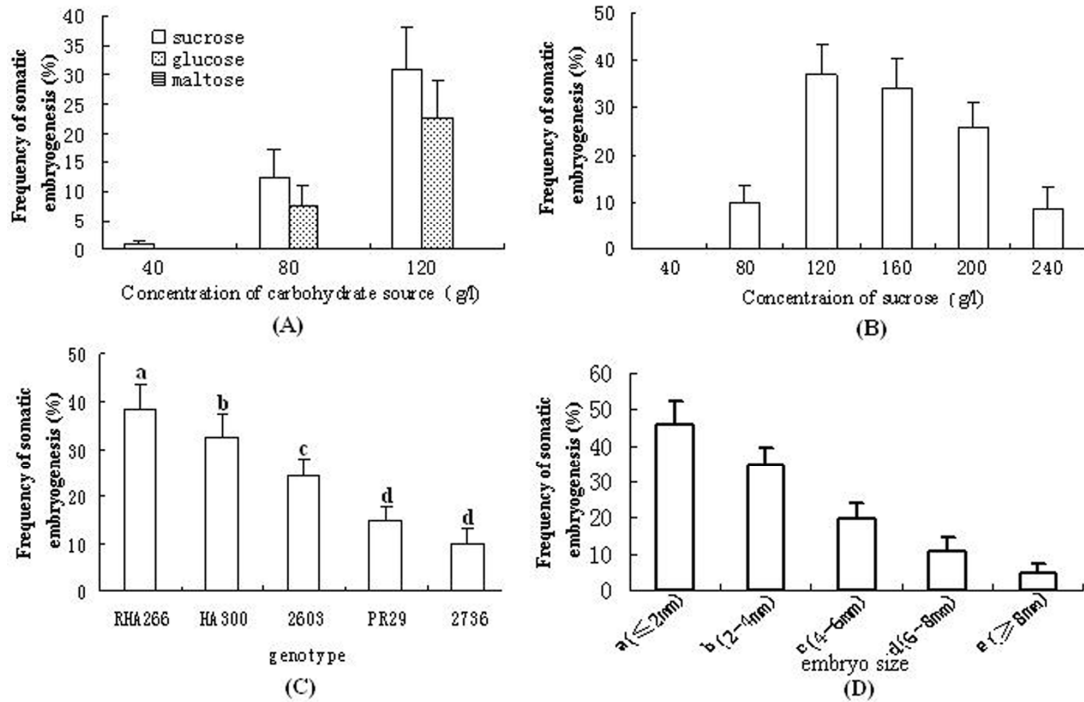
Fig 3. Effect of different variables on the frequency of somatic embryogenesis of sunflower. (A) Effect of different carbohydrate sources. Embryos were cultured on MS medium containing 0.4mg/l 2,4-D and different concentrations (40, 80, 120 g/l) of sucrose, glucose and maltose. (B) Effect of different concentrations of sucrose. Embryos were cultured on MS medium containing 0.4mg/l 2, 4-D and varying concentrations (40 - 240 g/l) of sucrose. (C) Effect of different genotypes. Embryos of different genotypes (RHA266, HA300, 2603, PR29, 2736) were cultured on MS medium containing 0.4mg/l 2, 4-D and 120 g/l of sucrose. (D) Effect of different embryo size. Different sizes of embryos (≤2mm, 2-4mm, 4-6mm, 6-8mm, ≥8mm) were cultured on MS medium containing 0.4mg/l 2, 4-D and 120 g/l of sucrose. Frequencies denoted by different letters are significantly different according to DMRT at α = 0.05.

A PCR detection of transformed sunflower
M: λDNA/EcoRI+HindIII
1,2,4: transformed plantlet
3: pseudo positive plantlet
5,6: untransformed plantlet
7: water
8: positive comparison

B Southern blot detection of transformed sunflower
M: λDNA/EcoRI+HindIII
1, 2, 7: transformed plants
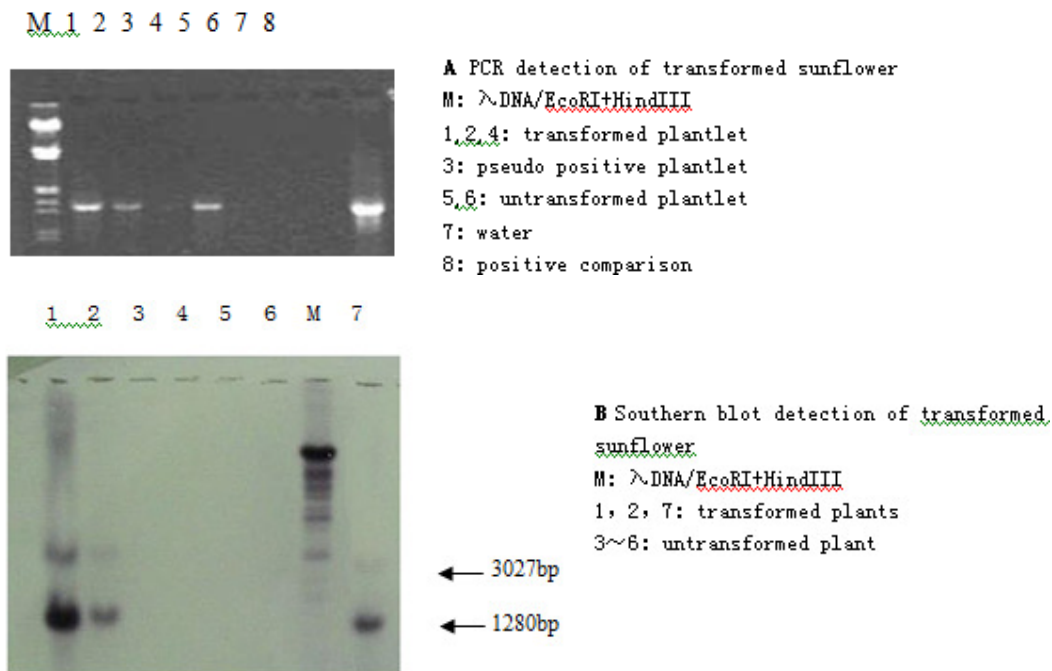3~6: untransformed plant

← 3027bp
← 1280bp

Fig 4. PCR and southern blot detection of transformed

medium. The immature embryos of sunflower were incubated for 20 min, then transferred to 50 ml glass containers with solidified MS medium and co-cultivated for 48 h at 25℃ in the dark. After co-cultivation, immature embryos were thoroughly washed in autoclaved tap water and placed on MS medium containing 0.4 mg/l 2, 4-D, 120 g/l sucrose, 30 mg/l kanamycin and 300 mg/l cefotaxime to induce embryogenesis. The explants were subsequently transferred into MS medium supplemented with 3-5% sucrose, 30 mg/l kanamycin and 300 mg/l cefotaxime for the germination of embryos. Germinated embryos were transferred into MS medium for shoot production.

PCR Detection

Genomic DNA was isolated from young leaf tissue of resistant sunflower plants using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma). The forward primer for LycB gene was 5′- GGATCCATGGATACTTTAGTGAAAACTC-CA-3′. The reverse primer was 5′-GGATCCGCTTATTCTT-TGTCCCGCAATAAGTT-3′. PCR reaction conditions (35 cycles in total) were: denaturation 1 min at 94℃, annealing 1 min at 55ºC, extension 1 min at 72ºC followed by a final elongation step of 5 min at 72ºC.

Southern blot

Genomic DNA was isolated from young leaf tissue using Qiagen DNeasy (Maxiprep) columns, digested with HindIII and EcoRI, separated in 0.8% agarose gels (15–20μg DNA per lane) and transferred onto Boehringer-Nylon membrane by vacuum blotting. The full-length coding sequence of LycB gene was labelled as a probe. For DNA labeling and detection, we used the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche). All further steps (hybridization, washes, chemiluminescent detection/CSPD) were carried out according to the manufacturer's instructions.

Data analysis

40 immature embryos were used in total for treatments, and each treatment was repeated three times. The number of explants initiating somatic embryos and the somatic embryos per explants was counted 5 weeks after culture. Data were analyzed using ANOVA and Duncan's multiple range test (α=0.05).

Acknowledgements

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