Screening Chinese soybean genotypes for *Agrobacterium*-mediated genetic transformation suitability[*]

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Abstract: The *Agrobacterium*-mediated transformation system is the most commonly used method in soybean transformation. Screening of soybean genotypes favorable for *Agrobacterium*-infection and tissue regeneration is the most important step to establish an efficient genetic transformation system. In this study, twenty soybean genotypes that originated from different soybean production regions in China were screened for transient infection, regeneration capacity, and stable transgenic efficiency. Three genotypes, Yuechun 04-5, Yuechun 03-3, and Tianlong 1, showed comparable stable transgenic efficiencies with that of the previously reported American genotypes Williams 82 and Jack in our experimental system. For the Tianlong 1, the average stable transformation efficiency is 4.59%, higher than that of control genotypes (Jack and Williams 82), which is enough for further genomic research and genetic engineering. While polymerase chain reaction (PCR), LibertyLink strips, and β-glucuronidase (GUS) staining assays were used to detect the insertion and expression of the transgene, leaves painted with 135 mg/L Basta could efficiently identify the transformants.

Key words: Soybean, *Agrobacterium*-mediated transformation, Genotypes, Stable transgenic efficiency

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1 Introduction

Soybean [*Glycine max* (L.)] is an important food and oil crop with more than 5 000 years of cultivation history in China. China was the largest soybean exporter in the world before 1996. Since then, commercial transgenic soybean production increased in the United States of America, Brazil, and Argentina. China has gradually become the world's largest soy-bean importer due to the high quality and low price of imported transgenic soybean. The soybean production has continued to decrease in China and the domestic soybean industry is facing an unprecedented crisis. Over the past decade, transgenic soybeans have become the most widely cultivated genetically modified crop in the world, accounting for 47% of the global biotech crop area (75.4 million hectares) in 2011 (James, 2012). Though transgenic technology is considered to be one of the most important ways to breed new varieties of soybean, transgenic soybean breeding is still in its infancy in China (Qiu *et al.*, 2007).

Particle bombardment-based transformation and *Agrobacterium*-mediated transformation are the two

predominant methods for plant transformation (Hinchee *et al.*, 1988; McCabe *et al.*, 1988). Particle bombardment-based transformation has some shortcomings; e.g., it requires expensive equipment and often results in transformants with complicated and multiple copies (Dai *et al.*, 2001; Shou *et al.*, 2004; Travella *et al.*, 2005). Contrastingly, *Agrobacterium*-mediated transformation is less expense, allows for easier manipulation, requires a lower transgene copy number, and results in higher stable gene expression (Dai *et al.*, 2001; Shou *et al.*, 2004; Travella *et al.*, 2005). Thus, the *Agrobacterium*-mediated transformation is widely used for the transformation of a large number of crops, such as soybean, maize, rice, cotton, and canola (Gelvin, 2003).

The efficiency of *Agrobacterium*-mediated plant transformation depends on the success of the plant-*Agrobacterium* interaction (Gelvin, 2000). Plant genotype, explants vigour, *Agrobacterium* strain, vector, selection system, and culture conditions are all important factors in *Agrobacterium*-mediated plant transformation (Cheng *et al.*, 2004). The common *Agrobacterium* strains include LBA4404, disarmed C58, EHA101, and their derivatives (Cheng *et al.*, 2004). The plant tissues used to produce transgenic soybean plants vary but include shoot meristems (McCabe *et al.*, 1988), embryogenic suspension cultures (Finer and McMullen, 1991), immature cotyledons (Parrott *et al.*, 1989), axillary meristematic tissue located in seedling cotyledonary-nodes (Hinchee *et al.*, 1988), and embryonic plant tissues used for commercial (Monsanto Technology LLC, 2008). The reasons that genotypes influence the transformation efficiency are complex. Overexpression of *AtHTA1* gene increased *Agrobacterium* transformation efficiency in *Arabidopsis* and rice, which demonstrated that plant genes could alter the transformation efficiency (Mysore *et al.*, 2000; Yi *et al.*, 2006; Zheng *et al.*, 2009). It has been well documented that genetic factors determine the susceptibility of soybean genotypes to *Agrobacterium*-infection and regeneration capacity in the transformation process (Meurer *et al.*, 1998; Donaldson and Simmonds, 2000; Paz *et al.*, 2004; Liu and Wei, 2005; Ying *et al.*, 2008). Therefore, screening soybean genotypes for the germplasm resources suitable to *Agrobacterium*-mediated genetic transformation has become the focus for optimizing the soybean transformation system and improving transformation efficiency.

Although great progress has been made in soybean transformation, there still remain many challenges. Our work has focused on screening the soybean genotypes that are currently grown in China for high transformation efficiency. The improved cotyledonary-node transformation method and vector pTF102 with β-glucuronidase (*GUS*) reporter gene were employed in this study (Paz *et al.*, 2004). Twenty soybean varieties originating from different soybean production regions in China have been screened for *Agrobacterium*-mediated genetic transformation. Four genotypes showed a high transient transformation efficiency and regeneration rate. The stable transformation efficiencies of Yuechun 04-5, Yuechun 03-3, and Tianlong 1 are all greater than 2% and comparable to the American control genotypes Williams 82 and Jack in our experimental conditions.

2 Materials and methods

2.1 Plant materials

Twenty-two soybean genotypes were used in this study. Among them, fifteen soybean cultivars are spring soybean varieties commonly planted in Northeast China, including Heihe 06, Heihe 19, Heihe 25, Heihe 38, Heihe 43, Heihe 48, Hefeng 25, Hefeng 35, Heinong 35, Heinong 37, Heinong 44, Heinong 48, Heinong 51, Heinong 58, and Ha 03-3. Zhonghuang 30 and Zhonghuang 39 are summer soybean varieties that originated from the Huanghuai region of China. Tianlong 1, Yuechun 03-3, and Yuechun 04-5 are southern spring soybean varieties. The well-studied American varieties Jack and Williams 82 were used as control genotypes.

2.2 Vector and *Agrobacterium* strain

The *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986) and the binary plasmid pTF102 (Fig. 1) (Frame *et al.*, 2002) were used in this study. The vector includes a phosphinothricin acetyl transferase (*bar*) gene from *Streptomyces hygroscopicus* that confers resistance to herbicide phosphinothricin, an intron-containing *GUS* gene, and a spectinomycin-resistant marker (*aadA*) gene for bacterial selection.
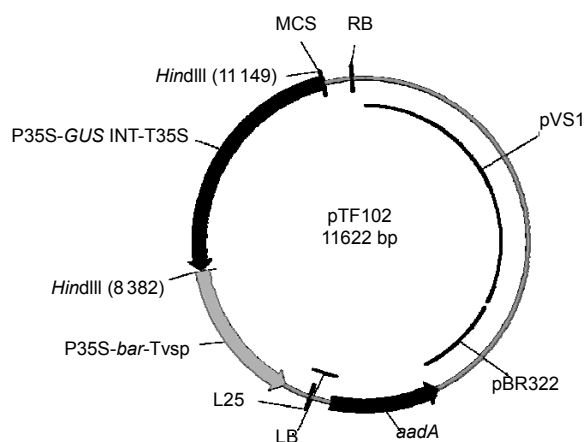
Fig. 1 Map of vector pTF102
P35S/T35S, CaMV 35S promoter/terminator; *GUS* INT, an intron-containing β-glucuronidase gene; *bar*, encoding phosphinothricin acetyl transferase; Tvsp, soybean vegetative storage protein terminator; *aadA* gene, a spectinomycin-resistant marker gene; pBR322, origin of replication for *Escherichia coli*; pVS1, origin of replication for *Agrobacterium*; LB, left border; RB, right border; MCS, multiple cloning site. *Hin*dIII is one of the unique restriction enzyme sites

Liquid YEP medium (10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract, pH 7.0) containing 50 mg/L kanamycin, 20 mg/L chloramphenicol, and 100 mg/L spectinomycin was inoculated with the *A. tumefaciens* EHA101/pTF102 and shaken at 28 °C (250 r/min) until the optical density at 650 nm (OD_{650}) reached 0.8–1.0. The *A. tumefaciens* culture was centrifuged (20 °C, 4000 r/min, 10 min) and the cell pellet was subsequently re-suspended in liquid co-cultivation medium (CCM) comprising 1/10 B5 salts and vitamins (Gamborg *et al.*, 1968), 3.9 g/L 2-[*N*-morpholino] ethanesulfonic acid (MES), 3% sucrose (30 g/L) (pH 5.4), filter-sterilized 0.25 mg/L gibberellic acid (GA_3), 400 mg/L L-cysteine, 1.67 mg/L *N*-6-benzylaminopurine (BAP), 154.2 mg/L dithiothreitol (DTT), and 200 μmol/L acetosyringone (As).

2.3 Explant preparation and inoculation

The cotyledonary-node method used in this study followed the procedure described by Paz *et al.* (2004). Briefly, soybean seeds were surface-sterilized by placing seeds into a tightly sealed chamber containing chlorine gas for 16 h. The sterilized seeds were soaked with sterile water at 28 °C under an 18-h photoperiod for 12–15 h. The imbibed soybean seeds were placed on sterile filter paper and cut longitudinally along the hilum. The cotyledons and hypocotyls (~5 mm) from a single seed were split evenly into two explants. Approximately fifty coat-removed explants were then inoculated in liquid CCM for 30 min. After inoculation, seven explants (adaxial side up) were randomly placed in Petri dishes (90 mm in diameter× 15 mm deep) on sterile filter paper placed on solid CCM containing 0.5% purified agar (Difco Agar, Noble), and incubated in the dark at 25 °C for 3–5 d.

2.4 Selection and plant regeneration

After co-cultivation, explants were then transferred into solidified shoot induction (SI) medium containing B5 salts and vitamins, 3% sucrose, 0.8% agar (8 g/L; Sigma, USA), 0.58 mg/L MES (pH 5.8), filter-sterilized 1.67 mg/L BAP, 250 mg/L ticarcillin (Tic), 100 mg/L cefotaxime (Cef), and 5 mg/L glufosinate, and incubated in a growth room at 28 °C under an 18-h photoperiod for two weeks. Then, the hypocotyl and shoots were trimmed from the explants and the remaining cotyledons with developing nodules were sub-cultured to fresh SI medium for another two weeks.

Four weeks after SI, the cotyledons were removed from the explants and the remaining tissues were transferred into shoot elongation (SE) medium. SE medium was composed of MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar (Sigma, USA), 0.58 mg/L MES, filter-sterilized 50 mg/L L-asparagine, 50 mg/L glutamine, 0.1 mg/L indole acetic acid (IAA), 0.5 mg/L GA_3, 1 mg/L zeatin riboside (ZR), 250 mg/L Tic, 100 mg/L Cef, and 5 mg/L glufosinate (pH 5.8). Explants were transferred to fresh SE medium every two weeks until the regenerated shoots were suitable for rooting. The incubation conditions were the same as the conditions for SI.

Elongated shoots (3–4 cm in length) were excised and placed into rooting medium (RM) containing MS salts and vitamins, 3% sucrose, 0.8% agar (Sigma, USA), 0.58 mg/L MES (pH 5.8), filter-sterilized 50 mg/L L-asparagine, 50 mg/L glutamine, 0.1 mg/L indole butyric acid (IBA), 250 mg/L Tic, and 100 mg/L Cef. After 1–2 weeks, the roots were fully developed to 2–3 cm in length, and eventually transplanted in the pots grown in the greenhouse.

2.5 Determination of the transient expression and regeneration rate

After 3 d of co-cultivation, the explants were first washed using sterile distilled water, and subsequently cut off at the upper side of the cotyledon (away from hypocotyl) and 3–5 mm below the cotyledonary node on the hypocotyl. The explants were harvested for GUS staining. According to the staining results, the explants were divided into three categories (strong, weak, and none). The transient rate of each category is calculated as: $n_e/n_t \times 100\%$, where n_e is the number of explants in each category and n_t is the number of total stained explants. The regeneration rate is defined as: $n_{ea}/n_{eb} \times 100\%$, where n_{ea} is the number of explants after four weeks screening and n_{eb} is the number of explants before screening.

2.6 Detection of transgenic soybean plants

Transgenic soybean plants were verified by leaf painting, polymerase chain reaction (PCR) analysis, GUS staining, and LibertyLink strip analysis. Leaf painting was performed following the procedure described by Ying *et al.* (2008) and Ma *et al.* (2012). Stock of LibertyLink® Basta (135 g/L) was diluted 1000-fold and painted on half the upper surface of tested soybean leaves. After 3 to 5 d, the painted leaves of the negative and positive plants died and remained healthy, respectively. For PCR analysis, genomic DNA of transgenic soybean was extracted using a simple and quick DNA extracting method (Edwards *et al.*, 1991). The 459-bp *bar* gene coding region was amplified using the pairs of primers: 5′-GTACCGGCAGGCTGAAGTCC-3′ and 5′-CGG TCTGCACCATCGTCAAC-3′. The amplified products were separated by electrophoresis on a 1% agarose gel (10 g/L) for about 20 min and photographed with a gel imaging system. For GUS staining, the leaves of transgenic soybeans were harvested, immediately submerged in GUS staining solution, and placed under a vacuum for 10 min (Jefferson *et al.*, 1987). The samples were incubated overnight in darkness at 37 °C. Chlorophyll was removed by submerging the tissue in 70% ethanol. LibertyLink strips (Envirologix, USA) were used to determinate genetically modified plants containing the phosphinothricin *N*-acetyltransferase (PAT) protein following the manufacturer's instruction. Briefly, a circular

leaf tissue was isolated by closing the cap of the Eppendorf tubes. The tissue was ground with a pestle for 20 to 30 s and 0.25 ml of protein extraction buffer was added before re-grinding. Development of the control line indicated that the strip had functioned properly. The second line (test line) would show up when the tested sample was positive.

2.7 Determination of the efficiency of soybean transformation

Positive transgenic seedlings were verified by PCR, leaf painting, GUS staining, and LibertyLink strip analysis. Transformation efficiency is defined as: $n_p/n_i \times 100\%$, where n_p is the number of positive transgenic seedlings and n_i is the number of explants initially infected.

2.8 Molecular analysis

Southern (1975) blot analysis was conducted on T_1 generations. Soybean genomic DNA was extracted from their leaf tissue using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). Approximately 30 µg of genomic DNA was completely digested with *Hin*dIII (Fermentas, Canada). The restriction fragments were separated on 0.8% agarose gel by electrophoresis and transferred onto a Hybond-N$^+$ nylon membrane (Amersham, UK). The PCR-fragments from the *bar* gene were labeled with Digoxigenin (DIG)-high prime according to the DIG High Prime DNA Labeling and Detection Starter Kit II instructions (Roche Applied Science, Germany). Prehybridization, hybridization, membrane washing, and detection were also conducted following the kit's instruction manual (Roche Applied Science, Germany).

3 Results

3.1 Transient infection efficiency and regeneration rate of soybean genotypes

Twenty soybean genotypes cultivated in different regions of China were collected for screening. First, we tested the susceptibility of these soybean genotypes to *Agrobacterium* infection by transient GUS staining. The major GUS staining tissues are cotyledonary nodes and hypocotyls, which are tissues easily accessible to the *Agrobacterium*. According to

the intensity of GUS staining, we divided the explants into three categories: strong, weak, and none (Fig. 2). The transient infection efficiencies were calculated and listed in Table 1. The control genotypes, Jack and Williams 82, showed 58.5% and 56.1%, respectively, of individuals expressing strong staining and less than 5% with no staining, indicating the effectiveness of *Agrobacterium* transient infection in our system.

For all the tested genotypes, the average percentage of individuals demonstrating strong staining was 19.5%, indicating low efficiencies of *Agrobacterium* infection for most genotypes. However, Tianlong 1, Yuechun 03-3, Yuechun 04-5, Hefeng 35, Hefeng 25, and Heinong 37 exhibited above-average percentages of strong staining. Moreover, these six genotypes all exhibited "no staining" percentages of less than 50%.

Table 1 Regeneration rate and transient GUS staining of explants infected by *Agrobacterium*

Genotype	Shoot regeneration		GUS staining						
	n_1	Regeneration rate[*] (%)	n_2	Strong		Weak		None	
				n_3	Ratio (%)	n_3	Ratio (%)	n_3	Ratio (%)
Heihe 06	539	13.4 ± 3.4^{jk}	40	1	2.5	17	42.5	22	55.0
Heihe 19	528	13.6 ± 1.9^{jk}	50	1	2.0	20	40.0	29	58.0
Heihe 25	613	23.8 ± 4.3^{hi}	55	0	0.0	19	34.5	36	65.5
Heihe 38	608	27.3 ± 2.1^{gh}	52	4	7.7	18	34.6	30	57.7
Heihe 43	553	25.7 ± 3.8^{gh}	40	4	10.0	25	62.5	11	27.5
Heihe 48	452	39.8 ± 6.5^{f}	59	0	0.0	24	40.7	35	59.3
Hefeng 25	289	21.1 ± 7.1^{hi}	37	9	24.3	16	43.2	12	32.4
Hefeng 35	370	13.5 ± 2.6^{jk}	40	11	27.5	13	32.5	16	40.0
Heinong 35	604	17.1 ± 3.6^{ij}	40	6	15.0	17	42.5	17	42.5
Heinong 37	12102	52.2 ± 1.1^{de}	44	9	20.5	16	36.4	19	43.2
Heinong 44	884	31.8 ± 7.2^{g}	40	4	10.0	15	37.5	21	52.5
Heinong 48	374	10.2 ± 3.6^{k}	40	4	10.0	21	52.5	15	37.5
Heinong 51	557	8.4 ± 3.2^{k}	40	7	17.5	20	50.0	13	32.5
Heinong 58	2027	58.7 ± 1.9^{d}	41	4	9.8	19	46.3	18	43.9
Ha 03-3	190	9.0 ± 2.7^{k}	40	0	0.0	6	15.0	34	85.0
Zhonghuang 30	400	20.8 ± 5.1^{hi}	39	0	0.0	22	56.4	17	43.6
Zhonghuang 39	340	24.1 ± 4.4^{h}	40	4	10.0	15	37.5	21	52.5
Tianlong 1	437	80.7 ± 4.3^{ab}	42	26	61.9	11	26.2	5	11.9
Yuechun 04-5	898	75.4 ± 3.2^{b}	40	16	40.0	18	45.0	6	15.0
Yuechun 03-3	355	51.5 ± 4.7^{e}	40	18	45.0	16	40.0	6	15.0
Jack	337	82.8 ± 3.3^{a}	41	24	58.5	15	36.6	2	4.9
Williams 82	356	67.7 ± 4.7^{c}	41	23	56.1	18	43.9	0	0.0
Average		34.9			19.5		40.7		39.8

[*] Regeneration rate was expressed as mean±standard deviation; means were compared using Fisher's least significant difference test, and different small letters showed significant difference ($P<0.05$). n_1: number of explants infected; n_2: total number of explants for GUS staining; n_3: number of explants
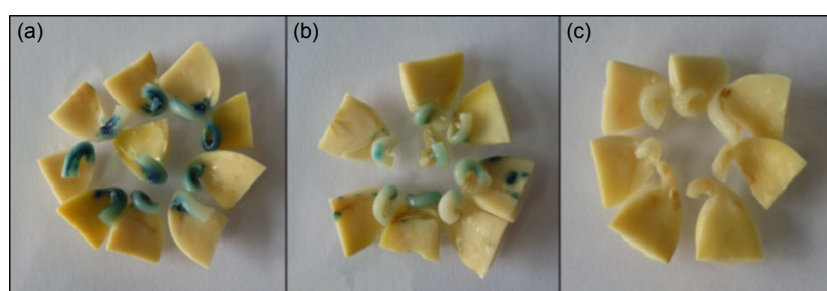

Fig. 2 Classification standards of GUS staining
(a) Strong, with deep dyeing in the cotyledon node; (b) Weak, with light dyeing in the cotyledon node or cotyledon; (c) None, with no dyeing

After selection with 5 mg/L glufosinate on SI medium for four weeks, healthy explants produced multiple observable buds (Fig. 3a). Some explants were dead and had no buds (Fig. 3b). Regeneration rate was calculated as the proportion of healthy explants for all the tested genotypes, with an average regeneration rate of 34.9%. As expected, Jack and Williams 82 varieties grew well and had 82.8% and 67.7% regeneration rates, respectively. Among the twenty genotypes originating from China, Tianlong 1, Yuechun 04-5, Heinong 37, Heinong 58, Yuechun 03-3, and Heihe 48 showed above-average regeneration rates.
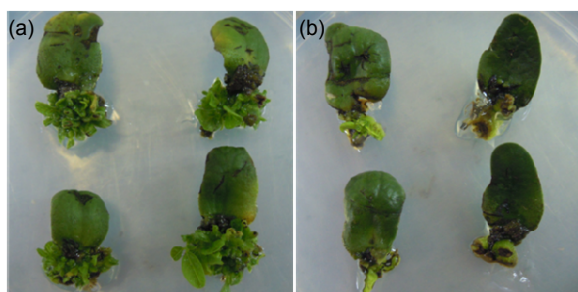

Fig. 3 Phenotype of explants after shoot induction (SI) for four weeks
(a) Subculture explants with a great of herbicide-resistant multiple buds; (b) Discarded explants with no or very little shoots

Based on transient infection and regeneration rates, Tianlong 1, Yuechun 04-5, Heinong 37, and Yuechun 03-3 performed better than other Chinese genotypes, and were used in the following stable transformation experiments.

3.2 Quick detection of T_0 transgenic plants

Due to the large number of T_0 transgenic plants that were generated in the lab, a rapid method for determining positive transgenic status was pivotal. PCR detection of a selectable marker gene is a traditional and effective way of doing this, though false positive results are sometimes observed. Therefore, we employed a two-step method for the identification of T_0 transgenic plants. First, the *bar* marker gene was detected by PCR in the T_0 transgenic plants, and the results indicated some might be positive events (Fig. 4a). Part of the PCR positive lines was randomly selected and tested using LibertyLink strips, which determined the PAT protein of transgenic plants

(Fig. 4b). We also used GUS staining to determine the positive transgenic plants (Fig. 4c). If 95% of the randomly selected plants were proven to be positive transgenic plants by test strips or GUS staining, we concluded that the PCR results were believable. When less than 95% of the randomly selected plants showed positive results by test strips or GUS staining, the two-step detection was repeated.
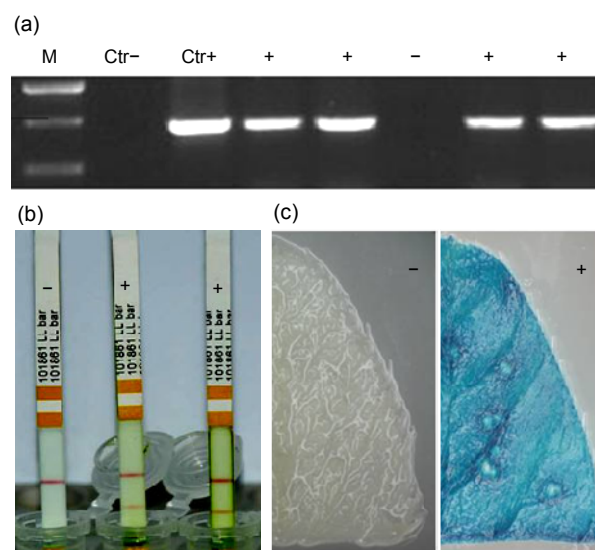

Fig. 4 Procedure for confirmation of T_0 transgenic plants
(a) PCR analysis of genome DNA of putative transgenic soybean plants using *bar* gene primers. The length of PCR production was 459 bp. M: DL2000 marker DNA; Ctr−: non-transformed soybean; Ctr+: plasmid DNA; (b, c) Events of the PCR positive lines were randomly selected and tested using LibertyLink strips (b) or GUS staining (c). +: positive transgenic soybean plant; −: negative transgenic soybean plant

To avoid the cumbersome procedure of PCR analysis and the expensive costs of the test strips and GUS staining, we employed leaf painting to detect transgenic soybean seedlings. Halves of the leaves were painted with 135 mg/L Basta. Five days later, painted half leaves of non-transgenic control seedlings were lesioned while the unpainted halves remained green (Fig. 5a). However, the whole leaves of the positive control plants with *bar* gene remained green (Fig. 5b). The T_0 seedlings selected by leaf painting were consistent with those of PCR and testing strips (data not shown). Thus, this method provides a low-cost, rapid, and accurate way to identify a large number of T_0 transgenic plants.

3.3 Stable transformation efficiencies of the screened soybean genotypes

The whole procedures of *Agrobacterium*-mediated cotyledonary-node transformation method used in this study were illustrated in Fig. 6. Under our conditions, the four Chinese genotypes tested (Tianlong 1, Yuechun 04-5, Yuechun 03-3, and Heinong 37) and the two control genotypes from the USA (Jack and Williams 82) produced positive transgenic plants. However, the stable transformation efficiencies varied among these genotypes. The average efficiencies of Tianlong 1 and Williams 82 were 4.59% and 4.39%, respectively, which were significantly higher than those of Yuechun 04-5, Yuechun 03-3, and Heinong 37 (Table 2). Yuechun 04-5 and Yuechun 03-3 showed similar average efficiencies to that observed in the Jack variety. The average efficiency of Heinong 37 was only 0.31%, the lowest among the tested genotypes (Table 2).

3.4 Analysis of T_1 seedlings

We examined T_1 progenies of five independent transformed T_0 lines with the integration of *bar* and *GUS* genes. All of the five independent transgenic plants produced positive T_1 seedlings. Chi-square analysis was performed on the segregation ratios of the five T_0 lines to determine goodness-of-fit for 3:1 and 15:1 (GUS^+:GUS^-). The results indicated that transgenic soybean Lines 1–4 were inherited in a Mendelian fashion and Line 5 was segregated in other ratio (Table 3). We further confirmed the copy number of inserted *bar* gene using Southern blots. Lines 1, 2, 3, and 5 had only one copy of the *bar* gene while Line 4 contained two copies (Fig. 7).

Table 2 Transformation efficiencies of six soybean genotypes

Genotype	n_1	n_2	Efficiency[*] (%)	RI
Heinong 37	5	1021	0.31±0.28[c]	+
Yuechun 03-3	5	1000	2.40±0.65[b]	++
Yuechun 04-5	5	1058	2.42±0.70[b]	++
Tianlong 1	5	1118	4.59±1.54[a]	+++
Jack	5	1014	2.84±1.25[ab]	++
Williams 82	5	1007	4.39±1.85[a]	+++

[*] Data of efficiency were expressed as mean±standard deviation; means were compared using Fisher's least significant difference test, and different small letters showed significant difference ($P<0.05$). n_1: number of experiments; n_2: number of explants infected; RI: recommendation index

Table 3 Segregation of *GUS* gene in the T_1 seedlings of five transgenic soybean lines

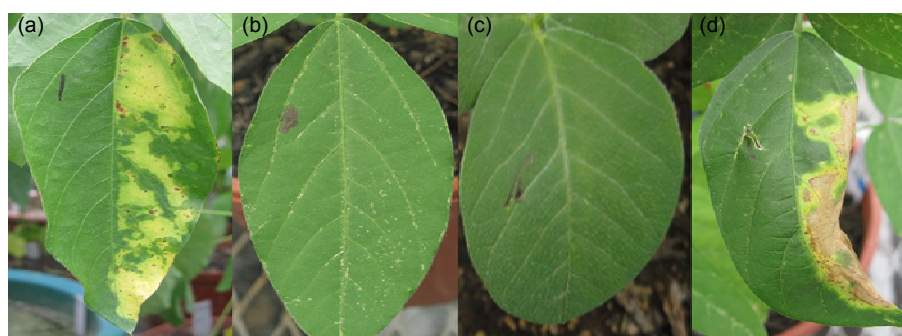| Transgenic line	Genotype	Number of T_1 plants	Segregation assay				
			GUS^+	GUS^-	Ratio	χ^2	P-value
Line 1	Yuechun 03-3	13	10	3	3:1	0.0256	0.8728
Line 2	Yuechun 03-3	18	12	6	3:1	0.2963	0.4142
Line 3	Yuechun 03-3	25	17	8	3:1	0.3333	0.3855
Line 4	Yuechun 03-3	10	10	0	15:1	0.0267	0.4142
Line 5	Yuechun 03-3	8	3	5	Other		

Fig. 5 Identification of the herbicide resistance of the leaves of transgenic soybean plants using 135 mg/L Basta
Half of a leaf with the black mark is not painted with herbicide, and the other side is painted with herbicide.
(a) Non-transgenic plant; (b, c) Positive transgenic plants; (d) Negative transgenic plant
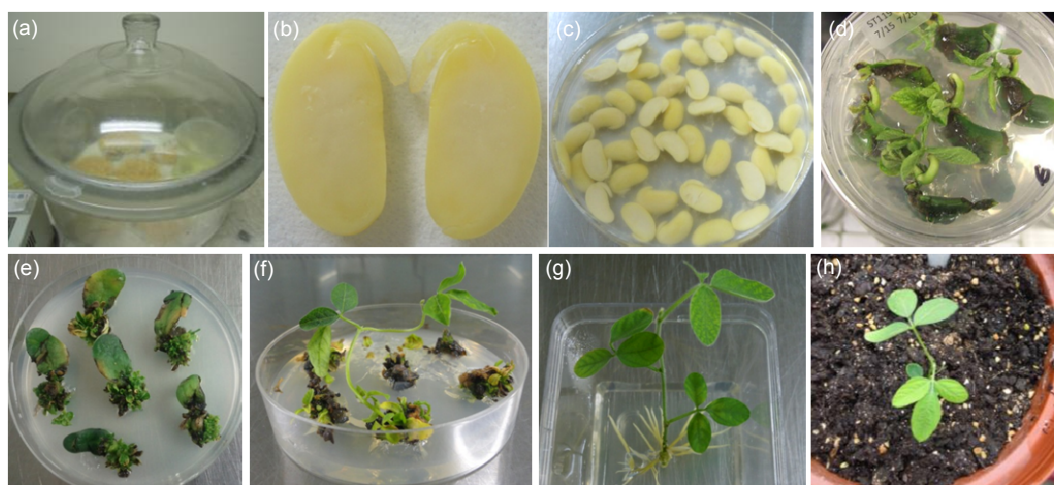
Fig. 6 *Agrobacterium*-mediated soybean transformation using the cotyledonary node as explants
(a) Surface sterilization of soybean seeds; (b) A longitudinal cut between the cotyledons and through the hypocotyl to generate two identical explants; (c) Inoculation of explants with *Agrobacterium*; (d) Two weeks after selection on shoot induction (SI) medium containing 5 mg/L glufosinate; (e) Four weeks after selection on SI medium containing 5 mg/L glufosinate; (f) Elongated shoot in shoot elongation (SE) medium; (g) Rooting of the resistant shoot; (h) A putative transgenic plantlet was transplanted to pots in the greenhouse
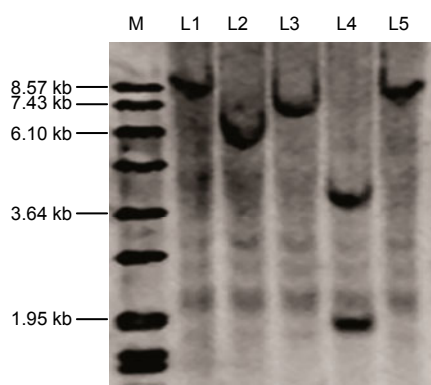

Fig. 7 Southern blot analysis of transgenic soybeans
Soybean genomic DNA was sampled from five independent events in the T_1 generation (L1, L2, L3, L4, and L5) and digested with the restriction enzyme *Hin*dIII, and hybridized with the *bar* probe labeled with DIG. A DIG-labeled DNA marker (M) was shown

4 Discussion

Soybean production in China has largely decreased in the past decade because of the high-quality and low price of imported transgenic soybean. Currently, Williams 82 and Jack soybean varieties are commonly used in international soybean transformation breeding and research. However, these two genotypes have poor agronomic traits and are only suitable to be grown in narrow regions. In order to speed up the process of transgenic soybean breeding in China, we screened soybean genotypes suitable for domestic ecological regions. In this study, twenty cultivated genotypes from different regions of China were collected and screened for their *Agrobacterium*-mediated cotyledonary node transformation.

Agrobacterium infection of plants is a process that resembled the pathogen-host interaction (Gelvin, 2003). Therefore, genotypes that are resistant to pathogen infection may have decreased susceptibility to *Agrobacterium* infection. In this research, we employed a transient transformation system to evaluate various genotypes' susceptibility to *Agrobacterium*. At the same time, another key factor of evaluation is the regenerability of explants. Although transient infection efficiency and regeneration rate were significantly different among the tested genotypes, Jack and Williams 82, the control genotypes in the study, showed a relatively good performance in both transient infection and regeneration processes. Therefore, our system is vigorous to screen the soybean genotypes for transgenic breeding. According to the transient infection efficiency and regeneration rates, four Chinese genotypes (Tianlong 1, Yuechun 03-3, Yuechun 04-5, and Heinong 37) showed potential for soy bean transformation. Though all of the four genotypes could stably produce transgenic soybean plants, the efficiencies varied. The Tianlong 1 variety showed the highest stable transformation efficiency among all tested genotypes. This suggests that

Tianlong 1 is suitable for further transgenic soybean breeding and functional genomics research. Yuechun 03-3 and Yuechun 04-5 also exhibited considerable transformation efficiencies and might be used in the future. Heinong 37 is a genotype with high regeneration rates and a good infection rate. However, using current protocol, the transformation efficiency was not as high as expected. Closely looking at the performance of the genotypes across the entire transformation process showed that the major obstacle to the transformation of a genotype is the elongation stage. Many putative transformed buds that initiated from the transformation process were not able to elongate. Further optimization of the hormone combination in SE medium may be necessary to improve transformation efficiency.

The method that we used for soybean transformation was a modified version of soybean cotyledonary node transformation. We observed that the cotyledonary nodes grew better when they were placed with the explants' adaxial side up. However, we did not observe a statistically significant difference between the two ways of placements of explants (abaxial or adaxial side up) on CC medium in terms of transformation efficiency. In most published soybean cotyledonary node transformation protocols, the embryonic axis in explants are excised to promote the growth of the axillary buds. However, removing the embryonic axis increased oxidative stress due to wounding. In this study, we kept the embryonic axis to reduce the oxidative stress. The growth of the embryonic axis can be suppressed by two weeks of high levels of selection. According to our observations, retaining the embryonic axis improved the initiation of axillary buds.

As a large amount of T_0 plants were generated during transgenic breeding, a quick and effective way to identify positive events was critical for subsequent growth and management of the seedlings. A two-step method, combining primary PCR testing and following marker gene detection, was employed to identify the positive T_0 transgenic plants in our system. However, the primary PCR testing is cumbersome and costly. Instead of PCR procedures, a leaf painting method was chosen (Ying *et al.*, 2008; Ma *et al.*, 2012). As leaf painting directly detected the effect of a selectable marker gene, it showed fewer false positive results than PCR procedures. Furthermore,

the leaf painting method was low-cost and easy to carry out.

Three Chinese soybean genotypes were selected for further applications in this study. The stable transformation efficiencies of these genotypes fulfill the requirements of soybean functional genomic research and transgenic breeding in China. However, these genotypes do not meet transformation efficiencies observed in commonly used North American genotypes. Further studies are needed to improve the soybean transformation efficiency of Chinese varieties and to optimize our transformation system.

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Compliance with ethics guidelines

Zhang-yue SONG, Jing-luan TIAN, Wei-zhe FU, Lin LI, Ling-hong LU, Lian ZHOU, Zhi-hui SHAN, Gui-xiang TANG, and Hui-xia SHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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