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Plant Science 173 (2007) 381–389
PLANT SCIENCE
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# An optimized *Agrobacterium*-mediated transformation for soybean for expression of binary insect resistance genes

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Received 11 February 2007; received in revised form 27 April 2007; accepted 13 June 2007
Available online 5 July 2007

**Abstract**

Here we described a method that used the soybean [*Glycine max* (L.) Merr] embryonic tip for *Agrobacterium*-mediated transformation. To improve transformation efficiencies, the effect of several factors were examined by measuring transient expression levels of β-glucuronidase and the number of resistant explants with PPT selection. The hypervirulent *Agrobacterium tumefaciens* strain KYRT1 proved to be a better transformer than EHA105 and LBA4404. Improved transformation efficiencies were obtained when embryonic tips were incubated with an *Agrobacterium* suspension ($A_{600}$ = 0.5) for 20 h. Optimized co-cultivation was performed in acidic medium (pH 5.4) at 22 °C in the dark for 5 days. By combining the best treatments, transgenic soybeans of seven cultivars were obtained that the *cryIA(c)* and *Pinellia ternata* agglutinins (*pta*) genes simultaneously presented. Most of the transgenic plants (about 80%) are fertile. The transformation frequency ([the number of PCR-positive regenerated plants/the number of infected explants] × 100) ranged from 4.29 to 18.0%. PCR and Southern analyses confirmed the stable integration of the binary insect resistance genes in the primary transgenic plants. The results of $T_1$ plants analysis showed the inheritance and stable integration of transgenes. Some transgenic soybeans ($T_1$) were proved to be high resistance to cotton bollworm by the insect resistance studies.


*Keywords:* *Agrobacterium tumefaciens*; Embryonic tip; Optimization; Soybean [*Glycine max* (L.) Merr]

## 1. Introduction

Soybean [*Glycine max* (L.) Merr] is one of the world's most important crops due to the high content of oil and protein in its seeds. Therefore, functional genomics efforts in soybean are focusing on seed traits [1]. Plant genetic transformation is a process whereby agronomically useful genes are directly introduced into important crops. The technique offers a significant tool in breeding programs by producing novel and genetically diverse plant materials. Transformation technologies have been applied to most crops. However, some hurdles remain to be overcomed before their application is practicable. Since the first reports of fertile transgenic soybean [2,3], various efforts have been made to solve problems associated with host/tissue specificity and low transformation efficiency. These include modifying the virulence of *Agrobacterium tumefaciens* strains [4–6], sonication of explant tissues to increase the number of infection sites [7,8], and addition of thiol compounds to the co-cultivation medium [9,10].

Two major modes of DNA delivery are currently utilized to transform soybean. One is particle bombardment of embryogenic tissue [8,11–14]. This technique often requires a prolonged tissue culture period to prepare target tissues and yields complex insertion patterns of transgenes into the plant genome. The other method involves *Agrobacterium*-mediated transformation of plant tissues [9,10,15–17]. A*grobacterium*-mediated transformation offers several advantages, such as defined integration of transgenes, preferential integration into transcriptionally active chromosomal regions, and potentially

*Abbreviations:* BA, 6-benzyladenine; *bar*, phosphinothricin acetyltransferase gene; GUS, β-glucuronidase; IBA, indole-3-butyric acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; PPT, phosphinothricin; *uidA*, β-glucuronidase gene from *Escherichia coli*; X-gluc, 5-bromo-4-chloro-3-indolyl-β-glucuronide

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doi:10.1016/j.plantsci.2007.06.010

Table 1
Media used for bacterial culture, tissue culture and soybean transformation

| Media | Composition |
|---|---|
| YEP | 10 g $l^{-1}$ peptone, 5 g $l^{-1}$ NaCl, 10 g $l^{-1}$ yeast extract, 1.5% (w/v) agar, pH 7.0 |
| PM | MS [24] salts and $B_5$ [25] vitamins, 30 g $l^{-1}$ sucrose, 3.5 mg $l^{-1}$ BA, 6% agar, pH 5.8 |
| Inf | 1/2 MS salts and $B_5$ vitamins, 30 g $l^{-1}$ sucrose, 10 g $l^{-1}$ glucose, 6.0 mg $l^{-1}$ BA, 0.5 g $l^{-1}$ MES, 200 μM acetosyringone |
| CM | Inf with 6% agar |
| SM | 1/2 MS salts and $B_5$ vitamins, 0.2 mg $l^{-1}$ BA, 0.2 mg $l^{-1}$ IBA, 30 g $l^{-1}$ sucrose, 300 mg $l^{-1}$ cefotaxime and various concentrations of PPT, 6.0 g $l^{-1}$ agar, pH 5.8 |
| RM | 1/2 MS salts and $B_5$ vitamins, 1.0 mg $l^{-1}$ IBA, 30 g $l^{-1}$ sucrose, 0.25 mg $l^{-1}$ PPT, 300 mg $l^{-1}$ cefotaxime, 8.0 g $l^{-1}$ agar, pH 5.8 |

a single or low copy number with relatively rare rearrangements [18]. The difficulties of soybean transformation focus on two aspects. One is its poor sensitivity to *Agrobacterium tumefaciens*. The other is the absence of regeneration system adapt to *Agrobacterium*-mediated transformation. We have established an efficient embryonic tip regeneration system and enhanced the transformation efficiency [16].

Effective resistance management strategies are essential in prolonging the usefulness of insect-resistant crops [19]. The bacterium *Bacillus thuringiensis* (Bt) has been used as a biological control agent against lepidopteran insects for more than 60 years. Significant progress has been made in Bt genetic engineering. *Pinellia ternata* is a traditional Chinese medicinal plant species in the *Araceae* family. Agglutinins of *P. ternata* (PTA) had significant insecticidal activities towards cotton aphids (*Aphis gossypii* Glover) and peach potato aphids (*M. persicae*) [20,21]. It is potential candidates for the control of aphids by genetic engineering [22].

The objective of this study was to identify some of the key parameters that enhance the efficiency of *Agrobacterium*-mediated transformation of soybean based on our previous work [16]. Also, we report the first instance of dual integration of *cryIA(c)* and *pta* in transgenic soybean.

## 2. Materials and methods

### 2.1. Plant material and media

Soybean [*Glycine max* (L.) Merr] cvs. Hefeng 25, Hefeng 35, Hefeng 39, Heinong 37, Heinong 43, Dongnong 42 and Lefeng 39 were used in these experiments. Mature, dry seeds were sterilized with chlorine gas for 8–10 h, as previously described [23]. The seeds were then soaked in distilled water for 24 h in the light at 28 °C. Afterwards, the cotyledons and primary leaves on the embryonic axes were excised to expose the meristem explant embryonic tips. The media used in this study are listed in Table 1.

### 2.2. Agrobacterium strains and plasmids

The following *Agrobacterium* strains were used in this study: KYRT1 [5], EHA105 [4] and LBA4404 [26]. pCAMBIA3301 (CAMBIA, Canberra, Australia), the binary vector used for optimization of the transformation system, contains the β-glucuronidase (GUS) gene (*uidA* coding region with an intron) as a visual marker and Bialaphos resistance gene (*bar*) that confers PPT resistance. This binary vector was transformed into the three *Agrobacterium* strains using the freeze–thaw method [27]. pCAMBIA3300 (CAMBIA, Canberra, Australia) contains the *bar*, *cryIA(c)* and *pta* genes (Fig. 1) that were used to create strains of insect-resistant soybean.

The EHA105 and LBA4404 *Agrobacterium* strains were maintained on solid YEP medium (Table 1) supplemented with 100 mg $l^{-1}$ kanamycin sulfate, while 100 mg $l^{-1}$ rifampicin was used for KYRT1. A single colony was transferred to 5 ml of YEP liquid medium containing the appropriate selective antibiotic, and the culture was shaken overnight at 200 rpm and 28 °C. The overnight bacterial solution was transferred into 50 ml of YEP selective medium. The culture was then grown overnight under the same conditions as described above. When the culture was in log phase, which corresponded to an absorbance at 600 nm ($A_{600}$) of 1.4–1.6, the cells were pelleted by centrifugation at 4000 rpm for 10 min and resuspended in infection medium (Inf, Table 1). This *Agrobacterium* suspension was used for infection.

### 2.3. System sensitivity to PPT and cefotaxime

Embryonic tips and shoots were cultured on $MSB_5$ medium (MS salts and $B_5$ vitamins) supplemented with 0.2 mg $l^{-1}$ BA and PPT (0, 0.5, 0.75, 1.0, 1.25 mg $l^{-1}$) or cefotaxime (100, 200, 300, 400, 500, 700 mg $l^{-1}$). All antibiotics were filter-sterilized and added to the autoclaved media after the latter had been cooled to 45 °C prior to solidification. After 4 weeks, the

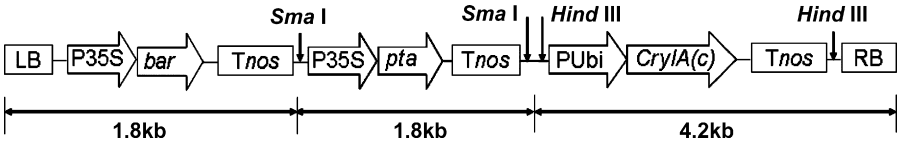


Fig. 1. Schematic representation of T-DNA regions of pCAMBIA3300 containing the *bar*, *pta* and *cryIA(c)* genes. LB/RB-left/right T-DNA border sequences; P35S – CaMV35S promoter; *bar* – coding region of the phosphinothricin acetyltransferase gene; Tnos – nopaline synthase terminator; *pta* – coding region of the *Pinellia ternate* agglutinins; PUbi – Ubiquitin promoter; *cryIA(c)* – coding region of the synthetic *Bacillus thuringiensis* insecticidal crystal protein gene.

adventitious shoots were counted and regeneration frequencies were determined.

### 2.4. Transformation

Embryonic tips (apical regions directed upwards, Fig. 2a) were cultivated on preculture media (PM, Table 1) for 24 h and used for transformation (Fig. 2b). The transformation process was divided into five sequential steps: bacterial inoculation, co-cultivation, resting, selection and plant regeneration. After preculture on PM, the explants were incubated in *Agrobacterium* suspension for 20 h. Approximately 15 explants were cultured on each petri dish (10 cm × 2 cm) containing 35 ml co-cultivation medium (CM). The petri dishes were sealed with Parafilm and placed in the dark for 5 days. For the resting step, the embryonic tips were placed on selection media (SM) without PPT and cefotaxime at 28 °C for 5–7 days. The explants were then cultured on SM with 0.5 mg $l^{-1}$ PPT for 20 days. Explants were subcultured on the fresh medium at 10-day intervals. Resistant explants were cultured on SM with 0.75 and 1.0 mg $l^{-1}$ PPT, one after the other for 3–4 weeks. When the height of the resistant shoots reached about 3 cm, they were transferred to rooting medium (RM) and cultured at 25 °C under a 16-h photoperiod with cool white fluorescent lights (80 μmol $m^{-2}$ $s^{-1}$). After 2–3 weeks, the regenerated plants with healthy roots were transferred to soil in pots and grown in the greenhouse.

### 2.5. Evaluation of factors influencing transformation

A range of parameters was evaluated using about fifty explants for each experiment. Each experiment was repeated three times. The parameters included the *Agrobacterium* strains, pH of the CM (5.0, 5.2, 5.4, 5.6, 5.8), and temperature of the co-cultivation period (19 °C, 22 °C, 25 °C, 28 °C). All of the parameters were evaluated and optimized on the basis of GUS activity of embryonic tips or the number of regenerating explants. The data were analyzed using analysis of variance (ANOVA) for a completely randomized design (CRD). Duncan's new multiple-range test (DMRT) was used to separate the means for significant effect.

### 2.6. Histochemical analysis of transient and stable GUS expression

The histochemical assay of GUS activity was carried out as described by Jefferson [28] using embryonic tips (after the 5-day co-cultivation) and resistant explants. Explants were incubated at 37 °C for 24 h in buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer (pH 7.0), 10 mM $Na_2EDTA$, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. T-DNA delivery was assessed by counting the number of embryos with GUS foci. Blue stained cells were visualized by sequentially soaking tissues in 75 and 95% (v/v) ethanol to remove chlorophyll before scoring the tissues as positive or negative for GUS expression.

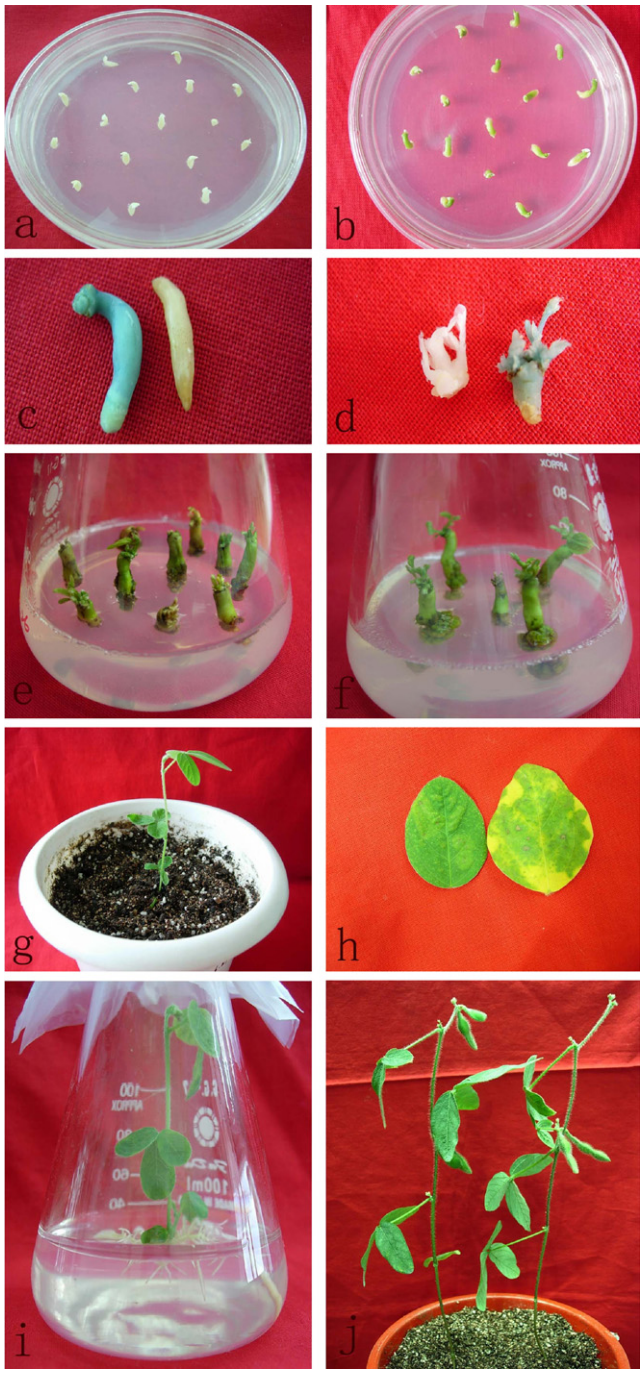


Fig. 2. *Agrobacterium*-mediated transformation of soybean from embryonic tips. (a) Embryonic tips on preculture medium. (b) Explants on preculture medium after 24 h. (c) Transient GUS expression in embryonic tip after co-cultivation for 5 days; an uninfected control is shown to the right. (d) Stable expression of the *gus* gene in an explant after 3 weeks in selection medium; the control is shown to the left. (e) Resistant explants on selection medium for 2 weeks. (f) Resistant explants after 5 weeks on selection medium. (g) A putative transgenic soybean growing in pot. (h) PPT spot paint test in leaves of untransformed (right) and transformed plant (left). (i) PPT-resistant plantlet with healthy roots grown on the rooting medium. (j) Mature transgenic plantlets in the greenhouse.

### 2.7. Herbicide leaf painting assay

Healthy leaves of non-transformed (control) and transformed ($T_0$) plants were selected for leaf painting. Using a

writing brush, 200 mg $l^{-1}$ PPT with 0.1% Tween-20 was applied to the upper surface of selected leaves. The leaves were scored for herbicide damage 10 days after application.

### 2.8. Polymerase chain reaction analysis

Putative transformants were screened by the polymerase chain reaction (PCR) for the presence of the *bar*, *cryIA(c)* and *pta* genes. DNA was isolated from leaf tissues by CTAB extraction. The 440-bp coding region of the *bar* gene was amplified using 20-bp oligonucleotide primers (5′-GCACCATCGTCAACCACTAC-3′ and 5′-TGAAGTCCAGCTGCCAGAAAC-3′). The 680-bp coding region of *cyrIA(c)* was amplified using 21-bp oligonucleotide primers (5′-ATGGACAACAACCCAAACATC-3′ and 5′-TAGAATCAGGACCCCAGACAC-3′). The 804-bp coding region of *pta* was amplified using 20-bp oligonucleotide primers (5′-ATGGCCTCCAAGCTCCTCCT-3′ and 5′-GCTTATTAATTCACCTTCTC-3′). PCR amplification reactions contained 50 ng of template DNA, 0.4 μM of each primer, 100 μM of a dNTP mixture, 1 × *Taq* DNA polymerase reaction buffer and 2 U *Taq* DNA polymerase (Takara, Dalian, China) in a 50-μl final volume. The amplification reaction was carried out using a Perkin Elmer 9700 thermal cycler (Foster City, CA) under the following conditions: one cycle of 94 °C for 5 min; 35 cycles of 94 °C for 1 min (denaturation), 58 °C for 1 min (annealing), 72 °C for 1 min (extension); and a final extension at 72 °C for 10 min (one cycle). DNA from a non-transformed (control) plant was included in the experiments. The amplified products were separated by electrophoresis in a 1.2% agarose gel and visualized with ethidium bromide.

### 2.9. Southern blots

Genomic DNA was extracted from the leaves of untransformed and transformed plants using the CTAB method. High-molecular-weight DNA (20–30 μg) was completely digested with *Hind*III or *Sma*I. Digested DNA fragments were separated by electrophoresis in a 0.8% agarose gel and transferred to a Hybond-N + nylon membrane (Amersham, Buckinghamshire, England). The 804-bp PCR fragment containing the *pta* coding region and the 680-bp PCR fragment containing the *cryIA(c)* coding region of pCAMBIA3300 were labeled with α-$^{32}$P-dCTP using a random primer DNA labeling system (Takara, China) and used as probes for hybridization. Prehybridization, hybridization and membrane washing were carried out according to the manufacturer's instructions. Hybridized membranes were exposed to Kodak XAR-5 film at −70 °C for 2–3 days.

### 2.10. Insect bioassay and progeny segregation analysis

Cotton bollworm [*Helicoverpa armigera* (H.)] was provided by Jiangsu Academy of Agriculture Sciences, Jiangsu province. The seeds from Southern-positive transgenic $T_0$ plants were planted in the greenhouse. Then the leaves were sampled and placed in the test tubes. A moist filter paper was placed inside the tube to keep the leaves in a humid condition. The 3-s larvae were used as challenging insect in each performance and were incubated at 26 °C in the dark. Leaves from non-transformed plants were used as controls. The maturity, weight and leaf damage were recorded on 6 days after infestation. Each treatment was replicated three times and got the means. Heredity of transgenic plants was also studied through insect bioassay.

## 3. Results

### 3.1. Factors influencing Agrobacterium-mediated soybean transformation

In order to optimize conditions for soybean transformation, the effects of several parameters known to influence *Agrobacterium*-mediated DNA transfer were compared.

*Agrobacterium* strains play an important role in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer. The number of GUS-expressing explants produced by a 5-day co-cultivation was used as a measure of gene transfer efficiency (Table 2; Fig. 2c). Although all three *A. tumefaciens* strains – KYRT1, EHA105 and LBA 4404 – were effective, KYRT1 consistently produced more GUS-positive explants than LBA 4404. For this reason, LBA 4404 was excluded from further use. KYRT1 and the hypervirulent strain EHA105 were more effective; however, transformation with KYRT1 yielded a higher efficiency (69.3%). The differences between the two strains were not evident at the transient expression stage. But the resistant shoots from KYRT1 were even more so than EHA105 (data not shown), so KYRT1 was used in all subsequent transformation experiments. Conversely, the frequency of transient GUS expression varied greatly of the three cultivars.

A co-cultivation medium with a pH of 5.4 was found to work best with respect to frequency of resistant regenerating explants

Table 2
Effects of *Agrobacterium* strain type on soybean transformation[a]

| *Agrobacterium* strains | Cultivars | No. of explants | No. of GUS-positive explants | Frequency of GUS expression (%) |
|---|---|---|---|---|
| KYRT1 | Hefeng25 | 51 | 35 | 68.6 |
| | Hefeng35 | 55 | 47 | 85.4 |
| | Dongnong42 | 50 | 27 | 54 |
| Means | | | | 69.3 |
| EHA105 | Hefeng25 | 48 | 29 | 60.4 |
| | Hefeng35 | 56 | 42 | 75 |
| | Dongnong42 | 53 | 26 | 49 |
| Means | | | | 61.5 |
| LBA4404 | Hefeng25 | 52 | 22 | 42.3 |
| | Hefeng35 | 46 | 25 | 54.3 |
| | Dongnong42 | 58 | 20 | 34.5 |
| Means | | | | 43.7 |

[a] Embryonic tips were incubated with *Agrobacterium* and co-cultivated (pH 5.8) at 25 °C. Each mean represents three replications.

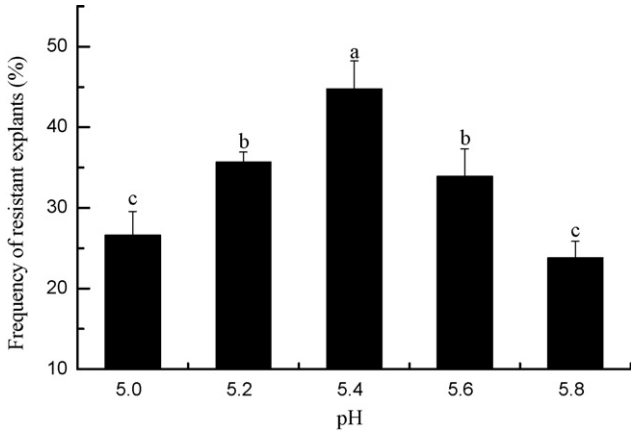


Fig. 3. Effect of co-cultivation medium pH on transformation efficiency of soybean. Embryonic tips of Hefeng 35 were incubated with *Agrobacterium* strain KYRT1 at 25 °C. Columns denoted by different letters are significantly different according to the Duncan's new multiple-range test at $p < 0.05$. Vertical bars represent the standard error.

(highest frequency was 44.8%). A decrease in transformation frequency occurred in co-cultivation medium that was either below or above this pH threshold value (Fig. 3).

With a view to determining the suitable temperature during co-cultivation, experiments were performed at temperatures ranging from 19 to 28 °C. The results (Fig. 4) showed that the highest frequency of resistant explants was observed at 22 °C, in which 95% of total embryonic tips showed GUS activity and 59.8% of them were PPT-resistant. The number of resistant explants markedly decreased when the temperature was decreased to 19 °C or increased to 25 °C. The lowest levels were observed at 28 and 19 °C.

### 3.2. Selection and regeneration

As sensitivity to PPT is another factor that affects the ability to produce transgenic soybeans, the effects of increasing

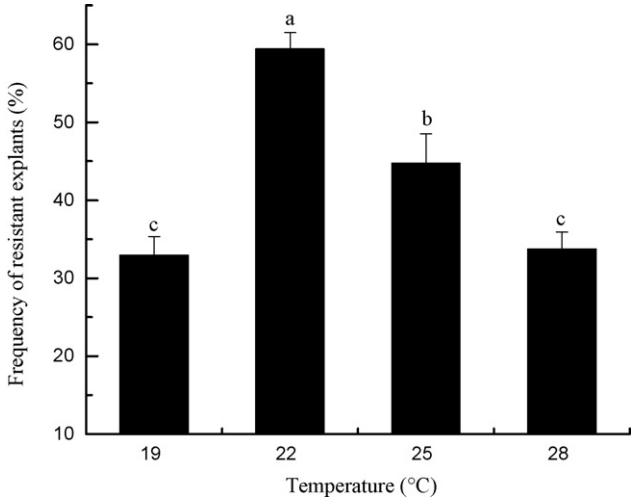


Fig. 4. The effect of co-cultivation temperature on transformation efficiency of soybean. Embryonic tips of Hefeng 35 were incubated with *Agrobacterium* strain KYRT1 at pH 5.4. Columns denoted by different letters are significantly different according to the Duncan's new multiple-range test at $p < 0.05$. Vertical bars represent the standard error.

Table 3
The percentage of surviving explants on selection medium supplemented with PPT[a]

| Cultivars | Explants | PPT concentrations (mg $l^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 0.75 | 1.0 | 1.25 |
| Hefeng25 | Embryonic tips | 68 | 6 | 3 | 1 | 0 |
| | Shoots | 25 | 2 | 0 | 0 | 0 |
| Hefeng35 | Embryonic tips | 75 | 7 | 4 | 2 | 0 |
| | Shoots | 32 | 2 | 1 | 0 | 0 |
| Dongnong42 | Embryonic tips | 69 | 5 | 2 | 0 | 0 |
| | Shoots | 28 | 3 | 1 | 0 | 0 |

[a] Each mean represents three replications.

concentrations of PPT were assessed separately on embryonic tips and *in vitro* developed shoots. The embryonic tip system was very sensitive to PPT. The tips almost died when treated with 1.0 mg $l^{-1}$ PPT and were unable to form any shoots when treated with 0.5 mg $l^{-1}$ PPT or more (Table 3). The shoots were more sensitive: survival percentage was only about 2% with 0.75 mg $l^{-1}$ PPT. So during the selection, the PPT concentrations increased gradually as 0.5, 0.75, 1.0 mg $l^{-1}$. A level of cefotaxime as high as 300 mg $l^{-1}$ was enough to restrain bacterial growth (data not shown).

The effective mode of selection use in this study produced a larger number of resistant embryonic tips and improved regeneration frequency. Stable GUS expression of resistant explants was also observed (Fig. 2d). The long-playing selection with higher selection pressure also eliminated many non-transformants (Fig. 2e and f). Overall, this selection system may be useful for the effective transformation and regeneration of transgenic plants. When the resistant shoots were as high as 3–4 cm, they were transferred to rooting medium and produced healthy roots (Fig. 2i). The potted regeneration plantlets proceeded to undergo normal development (Fig. 2g).

### 3.3. Transformation of multiple soybean elite lines

Using this optimized protocol, transgenic soybeans were obtained from the embryonic tips of seven elite lines. The frequency of plant regeneration ranged from 4.29 to 18.0% (Table 4). Genotypic differences were still observed in the seven cultivars. All of the PPT-resistant plantlets from each line were grown in a greenhouse. Almost all of the plants were normal in morphology and the majority (about 80%) produced seeds (Fig. 2j).

### 3.4. Analysis of putative transformants

Thirty putative transgenic plants derived from PPT-resistant embryonic tips from the seven elite lines were randomly selected and checked by PCR. Twenty-four of the plants showed the expected 440-bp band (for *bar* gene), 680-bp band (for *cryIA(c)* gene) and 804-bp band (for *pta* gene) (Fig. 5). These PCR results confirmed that most (approximately 80%) of the regenerated plants contained the transgenes derived from the pCAMBIA3300 plasmid. The production of negative plants

Table 4
Efficiency of *A. tumefaciens*-mediated transformation of soybean elite lines[a]

| Cultivars | Number of embryonic tips infected | Number of PPT-resistant explants | Regenerated plantlets | Produced PCR-positive plants | Frequency[b] (%) |
|---|---|---|---|---|---|
| Hefeng 25 | 38 | 14 | 6 | 5 | |
| | 47 | 28 | 9 | 7 | |
| | 40 | 22 | 7 | 5 | |
| Total | 125 | 64 | 22 | 17 | 13.6 |
| Hefeng35 | 62 | 35 | 11 | 9 | |
| | 43 | 28 | 8 | 6 | |
| | 38 | 21 | 7 | 7 | |
| Total | 143 | 84 | 26 | 22 | 15.38 |
| Hefeng39 | 34 | 18 | 7 | 6 | |
| | 41 | 25 | 9 | 6 | |
| | 45 | 30 | 11 | 8 | |
| Total | 120 | 73 | 27 | 20 | 16.67 |
| Heinong 43 | 70 | 25 | 5 | 4 | |
| | 63 | 21 | 6 | 3 | |
| Total | 133 | 46 | 11 | 7 | 5.26 |
| Heinong 37 | 61 | 19 | 5 | 3 | |
| | 79 | 23 | 7 | 3 | |
| Total | 140 | 42 | 12 | 6 | 4.29 |
| Dongnong 42 | 45 | 28 | 9 | 5 | |
| | 60 | 32 | 10 | 7 | |
| | 33 | 18 | 8 | 5 | |
| Total | 138 | 78 | 27 | 17 | 12.31 |
| Lefeng39 | 37 | 20 | 7 | 6 | |
| | 40 | 25 | 9 | 7 | |
| | 45 | 28 | 11 | 9 | |
| Total | 122 | 73 | 27 | 22 | 18.0 |

[a] All the embryonic tips were inoculated with *Agrobacterium* strain KYRT1 at $A_{600} = 0.5$ for 24 h and cocultivated (pH 5.4) at 22 °C for 5 days.
[b] (The number of produced PCR-positive plants/the number of the infected explants) × 100.

could be due to non-transformed shoots surviving in the selection medium or the unstable integration of the transferred gene into the plant genome.

Healthy leaves of plants that were PCR-positive for *bar*, *cryIA(c)* and *pta* were painted with 200 mg $l^{-1}$ PPT. All of the plants showed resistance to PPT painting, while the non-transformed controls showed necrosis (Fig. 2h). The result verified the functional expression of *bar* genes in the transgenic plants.

Six randomly sampled PCR-positive for *bar*, *cryIA(c)* and *pta* plants were further subjected to Southern blot analysis. Both *cryIA(c)* and *pta* were detected (Fig. 6) in the $T_0$ plants analyzed, whereas no hybridization signal was detected in the non-transformed plants. The number of hybridization bands reflected the number of insertion loci of the transgenes in the plant genome. Four of the six plants contained a single *cryIA(c)* and *pta* gene. The frequency of single inserts was about 66.7%. Two plants showed two *cryIA(c)* and *pta* gene loci. The results of the Southern analysis were in accordance with those of the PCR analysis and PPT-resistance analysis, thus confirming the presence, integration and expression of the *cryIA(c)* and *pta* genes in the transformants.

### 3.5. Inheritance of transgenes and insect bioassay

To confirm the inheritance of the transgenes to the next generation. DNA was extracted from the $T_1$ progeny of transformants $T_0$-1, $T_0$-3, $T_0$-6 shown in (Fig. 6), and analyzed by PCR. The *cryIA(c)* and *pta* gene were present in the PPT-resistant progeny and absent from the sensitive, negative one (Fig. 7).

Different transgenic lines showed the diversitiy of insect resistance. The leaves of the highest resistance were hardly any eaten, the control was completely destroyed, and some of the transgenic plants were placed in the middle or as the control (Fig. 8). The effects of *cryIA(c)* transgene expression on the development of cotton bollworm larvae were recorded 6 days after infestation. Compared to non-transgenic control plants, larvae growth that fed on the leaf from transgenic plants was evidently restrained, which represented less body, higher mortality and lower weight. The results of the leaf bioassay were summarised in Table 5. These results showed the transgene was expressed in the transgenic soybeans and some had high resistant to insect.

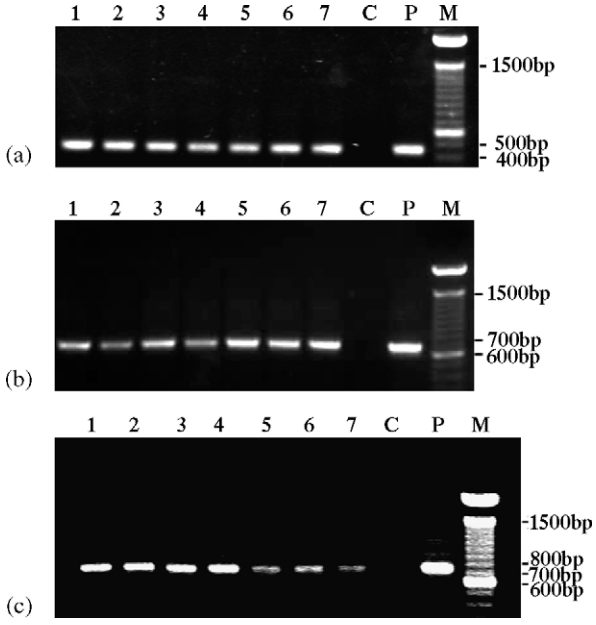


Fig. 5. Representative PCR analysis of genomic DNA to detect the presence of the *bar*, *cryIA(c)* and *pta* genes in putative transgenic soybean plants. (a) PCR amplification of the 440-bp fragment of the *bar* gene. (b) PCR amplification of the 680-bp fragment of the *cryIA(c)* gene. (c) PCR amplification of the 804-bp fragment of the *pta* gene. *Lane* M: Molecular weight marker; *Lane* P: pCAMBIA3300 plasmid DNA (positive control); *Lane* C: DNA from untransformed plant (negative control); *Lanes* 1–7: DNA from independently transformed plants.

The insect resistance of $T_1$ progeny occurred to be segregative. The progeny of the selected transgenic lines showed inheritance of the transgene in a Mendelian manner(Table 6). A segregation ratio of 3:1 was observed in two lines ($T_0$-3 and $T_0$-6). A line ($T_0$-1) showed a segregation pattern of 15:1. These results accorded with the Southern analysis of $T_0$ and indicated the stable inheritance of transgene to the progeny.

## 4. Discussion

Reports on a number of crop plants have suggested that *Agrobacterium* strains differ with respect to their capacity to transform tissues. The hypervirulent strain EHA105 has been commonly used for soybean transformation [8,10,16]. Meurer et al. [6] reported that strain KYRT1 significantly increased

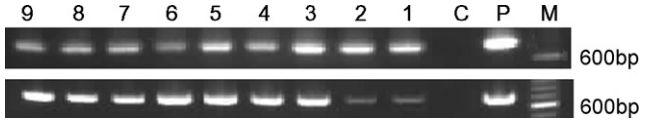


Fig. 7. PCR analysis of the $T_1$ progeny of transformed plants $T_0$-1, $T_0$-3, $T_0$-6 (shown in Fig. 6) to detect the presence of *pta* gene (up) and *cryIA(c)* (down). *Lane* M: Molecular weight marker; *Lane* P: pCAMBIA3300 plasmid DNA (positive control); *Lane* C: DNA from untransformed plant (negative control); *Lanes* 1–3: DNA from the $T_1$ progeny of $T_0$-1; *Lanes* 4–6: DNA from the $T_1$ progeny of $T_0$-3; *Lanes* 7–9: DNA from the $T_1$ progeny of $T_0$-6.

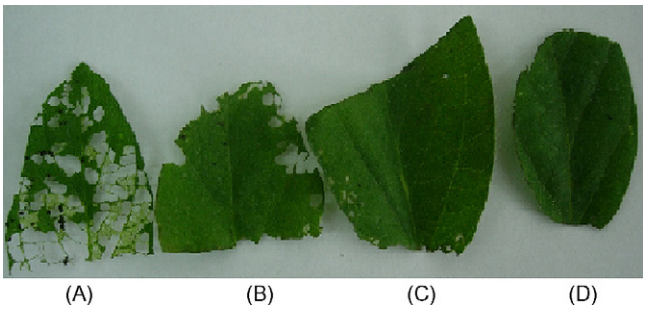


Fig. 8. Bioassay on transgenic soybean. (A) Untransformed control leaf; (B–D) Leaves from transgenic soybeans.

stable transformation over EHA105; but they also showed that KYRT1 had a significant decrease relative to EHA105 in shoot production of cotyledonary node transformation. However, in this study KYRT1 was more effective than EHA105 and LBA4404 in producing shoots. It also appeared that the use of KYRT1 can overcome the genotypic resistance found in some soybean cultivars, which may be due to the efficiency of this regeneration system. Recently, Ko et al. [29,30] observed that KYRT1 induced *Agrobacterium*-mediated transgenic somatic embryos at a high frequency. A similar result was found in *Agrobacterium*-mediated pea transformation [31].

The effect of co-cultivation medium pH on transformation efficiency is well documented. Mondal et al. [32] observed that pH 5.6 had positive effects on the transformation of tea. Shrivastava et al. [33] and Husnain et al. [34] found a pH of 5.6 to be the most effective for transforming *Cajanus cajan* and *Cicer arietinum*, whereas Meurer et al. [6] found a pH of 5.5 to be optimal for transforming *Glycine max*. A pH of 5.5 is generally considered to be suitable as acidic pHs may induce the vir (virulence) genes. Acetosyringone is known to activate

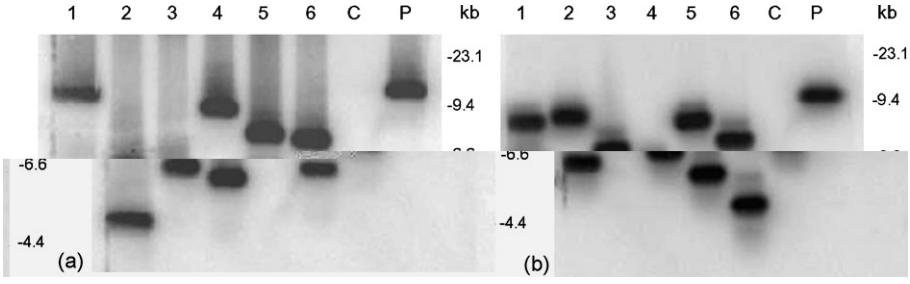


Fig. 6. Southern blot analysis of transformed soybeans ($T_0$ generation). *Lane* P: Plasmid DNA of pCAMBIA3300; *Lane* C: DNA from non-transformed plant of Hefeng35; *Lanes* 1–6: DNA from transformed plants. (a) Total $T_0$ genomic DNA was completely digested with *Sma*I and hybridized with an $\alpha$-$^{32}$P-labled *cryIA(c)* probe. (b) Total $T_0$ genomic DNA was completely digested with *Hind*III and hybridized with an $\alpha$-$^{32}$P-labled *pta* probe.

Table 5
Resistance assay on cotton bollworm in transgenic soybeans[a]

| No. of plant[b] | Mortality (%) | Average weight (mg) | Leaf damage[c] |
|---|---|---|---|
| CK1 | 25.4 | 13.86 | 4 |
| CK2 | 23.7 | 15.62 | 4 |
| 1 | 92.6 | 0.94 | 0 |
| 2 | 47.2 | 6.32 | 3 |
| 3 | 74.6 | 3.54 | 1 |
| 4 | 87.0 | 2.38 | 2 |
| 5 | 67.9 | 5.42 | 2 |
| 6 | 81.5 | 3.34 | 1 |

[a] The data were set out after 6-day resistance assays.
[b] CK1–CK2 represented untransformed control plants, 1–6 represented transgenic soybeans.
[c] Degree of leaf damage was determined according to leaf damage 0, 1, 2, 3 and 4 represented leaf mildestly damage, mildly damage, moderately damage, severely damage and completely destroyed, respectively.

the virulence genes of the Ti plasmid at pH 5.0–5.5, and to initiate the transfer of T-DNA. In the present study, it appears that the pH of the co-cultivation medium (pH 5.4) favored the induction of the *Agrobacterium tumefaciens* vir genes and might have contributed to the high efficiency of transgenic soybean plants.

Lowering the co-cultivation temperature has been shown to improve *Agrobacterium*-mediated gene transfer to plant cells. In a report on cotton transformation [35], co-cultivation of cotyledon discs at 21 °C, compared to 25 °C, consistently resulted in higher transformation frequencies. Dillen et al. [36] indicated that temperature played an important role in transformation with *Agrobacterium tumefaciens*. In their study, the best transformation efficiency was obtained at 22 °C in both *Phaseolus acutifolius* and *Nicotiana tabacum*, irrespective of the type of helper plasmid. We found the optimal temperature for co-cultivation is 22 °C. Low temperatures promoted pilus assembly, leading to an increased number of pili on the cell surface. It may be that the lower temperature resulted in enhanced functioning of the VirB-VirD4 part of the T-DNA transfer machinery [37].

In this report, a reliable procedure for soybean transformation has been optimized. This protocol is characterized by a high efficiency of transformation, and is applicable to multiple elite soybean cultivars. Also, transgenic soybeans were obtained with binary insect resistance by confirming the presence, integration of the *cryIA(c)* and *pta* genes in the transformants. The inheritance of transgene was verified in a Mendelian manner by the analyses of $T_1$ progeny. Also some transgenic showed high resistance to insect. Therefore, this system provides an effective approach to soybean transformation.

Table 6
Segregation of transgene in $T_1$ progeny of some transgenic lines[a]

| Transgenic lines | No. of plants tested | No. of IRP | No. of NIRP | Expected ratio | $\chi^{2b}$ |
|---|---|---|---|---|---|
| $T_0$-1 | 40 | 36 | 4 | 15:1 | 0.96 |
| $T_0$-3 | 35 | 23 | 12 | 3:1 | 1.61 |
| $T_0$-6 | 48 | 38 | 10 | 3:1 | 0.44 |

[a] IRP: insect-resistant plant; NIRP: non-insect-resistant plant.
[b] Tabulated $\chi^2$-value at 5% probability for 1 degree of freedom is 3.84. The calculated $\chi^2$-value is less than the $\chi^2$-table value. The progeny plants showed a Mendelian segregation ratio (3:1).

## Acknowledgements

We are grateful to Professor G. Collins for providing us with *Agrobacterium* strain KYRT1, also we thank Professor TL Wu for providing us with the vector pCAMBIA 3300 containing the binary insect-resistant genes. This work was supported by the National High-tech ''863'' Program of China (No. 2001AA244011).

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