



# Optimization of *Agrobacterium*-Mediated Transformation in Soybean

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High transformation efficiency is a prerequisite for study of gene function and molecular breeding. *Agrobacterium tumefaciens*-mediated transformation is a preferred method in many plants. However, the transformation efficiency in soybean is still low. The objective of this study is to optimize *Agrobacterium*-mediated transformation in soybean by improving the infection efficiency of *Agrobacterium* and regeneration efficiency of explants. Firstly, four factors affecting *Agrobacterium* infection efficiency were investigated by estimation of the rate of GUS transient expression in soybean cotyledonary explants, including *Agrobacterium* concentrations, soybean explants, *Agrobacterium* suspension medium, and co-cultivation time. The results showed that an infection efficiency of over 96% was achieved by collecting the *Agrobacterium* at a concentration of  $OD_{650} = 0.6$ , then using an *Agrobacterium* suspension medium containing 154.2 mg/L dithiothreitol to infect the half-seed cotyledonary explants (from mature seeds imbibed for 1 day), and co-cultured them for 5 days. The *Agrobacterium* infection efficiencies for soybean varieties Jack Purple and Tianlong 1 were higher than the other six varieties. Secondly, the rates of shoot elongation were compared among six different concentration combinations of gibberellic acid (GA<sub>3</sub>) and indole-3-acetic acid (IAA). The shoot elongation rate of 34 and 26% was achieved when using the combination of 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA for Jack Purple and Tianlong 1, respectively. This rate was higher than the other five concentration combinations of GA<sub>3</sub> and IAA, with an 18 and 11% increase over the original laboratory protocol (a combination of 0.5 mg/L GA<sub>3</sub> and 0.1 mg/L IAA), respectively. The transformation efficiency was 7 and 10% for Jack Purple and Tianlong 1 at this optimized hormone concentration combination, respectively, which was 2 and 6% higher than the original protocol, respectively. Finally, GUS histochemical staining, PCR, herbicide (glufosinate) painting, and QuickStix Kit for Liberty Link (*bar*) were used to verify the positive transgenic plants, and absolute quantification PCR confirmed the exogenous gene existed as one to three copies in the soybean genome. This study provides an improved protocol for *Agrobacterium*-mediated transformation in soybean and a useful reference to improve the transformation efficiency in other plant species.

**Keywords:** *Agrobacterium*, efficiency, half-seed explants, infection, regeneration, shoot elongation, soybean transformation

## INTRODUCTION

Soybean [*Glycine max* (L.)] is one of the most important oil crops and a significant source of protein for food and feed in the world. Soybean seeds are not only rich in essential amino acids, but also rich in dietary minerals, vitamins, unsaturated fatty acids, and isoflavones, which are implicated as beneficial food for human health (Han et al., 2003). Genetically modified (GM) soybean is one of the earliest introduced GM crops for commercial cultivation and the largest GM crop in terms of acreage planted worldwide (Yang et al., 2012). Studies have shown that the new GM crop varieties with important application values must be selected from hundreds, thousands, or even tens of thousands transformation events (Wang et al., 2006). On the other hand, although the soybean genome sequence has been released (Schmutz et al., 2010), the functions of most soybean genes are unknown. Therefore, an efficient and stable genetic transformation method is an important prerequisite to study gene functions in soybean and develop new soybean varieties by molecular breeding.

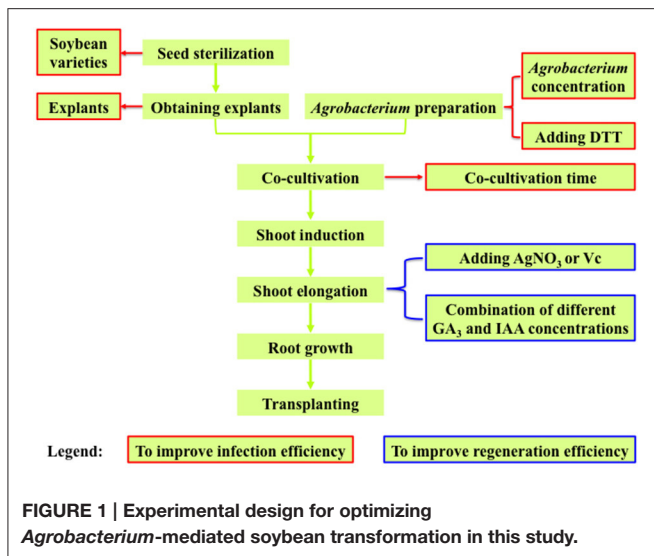
There are three main ways to deliver foreign DNA into host plants, including *Agrobacterium*-mediated transformation (Hinchee et al., 1988), particle bombardment (McCabe et al., 1988), and pollen-tube pathway method (Shou et al., 2002). *Agrobacterium tumefaciens* is a gram-negative bacterium and widespread in soil. *Agrobacterium* can infect plants through its Ti plasmid, and its T-DNA can be integrated to the host plant genome and inherited by the offspring of host plant (Wu et al., 2005). Particle bombardment method can insert the exogenous DNA (which is attached to micron-sized metal particles) directly into the tissue cells using high-pressure helium gas (Liu et al., 2001). Due to the strong penetrating power of small particle, the exogenous DNA-metal particle can penetrate the cell wall and cell membrane into cells. Particle bombardment transformation makes the transfer of DNA is no longer restricted by species or genotypes, especially suitable for the species and genotypes which are not sensitive to *Agrobacterium* infection (Wang et al., 2002). However, the cost of particle bombardment transformation is high, and gene rearrangement and high copy numbers are often observed in transgenic plants using this method (Wang et al., 2009). Pollen-tube pathway method delivers the exogenous DNA into a zygote cell or early embryo cells of recipient plant through the pollen tube directly (Zhou et al., 1983), which does not need tissue culture process (Ren et al., 2012), and exogenous DNA can be transferred to host plants directly without vector construct (Xiao et al., 2007). But the exogenous gene needs to go through many barriers along pollen tube into the zygote, and can be destroyed by the nuclease in stigma. Therefore, it is difficult to get a stable transformation rate by this method (Shou et al., 2002; Dong et al., 2011).

About 85% of the transgenic plants are obtained using the *Agrobacterium*-mediated transformation method (Yu et al., 2010). *Agrobacterium*-mediated transformation is the best choice for plant transformation due to its simple operation, high reproducibility, low copy number, and low experimental cost. This method also can transfer a large fragment of foreign gene into the host plant genome. *Agrobacterium*-mediated

transformation is also a preferred method in soybean. Hinchee et al. used soybean cotyledonary nodes as the explants to obtain transgenic soybean plants (Hinchee et al., 1988). From then on, many researchers used cotyledonary nodes as the explants for transformation (Di et al., 1996; Zhang et al., 1999). In addition to cotyledonary nodes, many other tissue types can be used as explants, such as primary leaf nodes, epicotyls, and hypocotyls, immature embryos, axillary buds, and stem tips. But the regeneration efficiency differed greatly among different explants. Cotyledonary nodes have a higher regeneration efficiency than other types of explants in soybean (Kim et al., 1990; Sato et al., 1993; Liu et al., 2004; Zhong and Que, 2009). In addition, using cotyledonary nodes as the explants has several other advantages. First, it is easy to obtain the cotyledonary nodes by germinating soybean seeds, which is not limited by the season. Second, the regeneration process is simple, including shoot induction, elongation and root induction. However, the transformation rate of this method is too low. Instead of germinating the soybean seeds to get cotyledonary nodes, Paz et al. imbibed mature soybean seeds for about 24 h to obtain the “half seeds” as the cotyledonary explants (Paz et al., 2006). The overall average transformation efficiency was 3.8% using this method, which was 1.5 times higher than using the cotyledonary nodes from the 5- to 7-d-old seedlings (Paz et al., 2006), but still low compared with the transformation efficiency in other crops such as rice 23% (Lin and Zhang, 2005; Ge et al., 2006) and maize 30–40% (Ishida et al., 1996; Yang et al., 2006). The previous study indicates that the low shoot elongation rate during explants regeneration is a bottleneck for soybean transformation (Song et al., 2013).

During the transformation process, there are many factors affecting the efficiency. The general transformation process includes the following steps: obtain the explants after seed sterilization; infect explants with the *Agrobacterium* suspension liquid and co-cultivate them on the co-cultivation medium (CCM) with their adaxial side (flat side) upwards (Gao et al., 2015); transfer the *Agrobacterium*-infected explants to shoot induction medium (SIM); transfer the explants to shoot elongation medium (SEM); put the elongated shoots into rooting medium; eventually the plants were transferred to pots and grown to maturity (Figure 1). During this process, many factors such as type of explants, concentration of *Agrobacterium* for infection, co-cultivation time, and medium composition (including CCM, SIM, SEM, and rooting medium), will affect the transformation efficiency. The efficiencies of regeneration and transformation also varied among different soybean genotypes (Hinchee et al., 1988; Bailey and Parrott, 1993; Donaldson and Simmonds, 2000; Yang et al., 2016). The overall transformation efficiency depends on the efficiencies of *Agrobacterium* infection and explant regeneration.

*Agrobacterium* concentration is an important factor affecting its infection efficiency (Paz et al., 2006). *Agrobacterium* concentration reflects its growth status and the *Agrobacterium* during logarithmic growth phase is thought to have higher infection ability (Zhou et al., 2011). In the *Vanda Kasem's Delight Orchid*, the highest  $\beta$ -glucuronidase (GUS) expression in protocorm-like bodies was observed when the optical density at 600 nm (OD<sub>600</sub>) of *Agrobacterium* suspension was 0.8



(Gnasekaran et al., 2014). In groundnut, the most suitable  $OD_{600}$  of *Agrobacterium* for infection was determined as 1.8 (Tiwari et al., 2015). The *Agrobacterium* concentration for infection of ramie was optimized as  $OD_{600} = 0.6$  (An et al., 2014).

Plant hormones play important roles in explant regeneration during tissue culture, especially auxins, cytokinins, and gibberellins. Auxin was discovered as a phytohormone with the chemical structure of indole-3-acetic acid (IAA), and synthetic auxins such as indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) also have auxin activity, which promote cell elongation, plant growth, and development (Normanly, 1997). Cytokinins promote cytokinesis, differentiation and growth of various tissues (Letham, 1967). 6-benzylaminopurine (6-BA), kinetin (KT), zeatin (ZT) are synthetic cytokinins. In plant tissue culture, the concentrations of auxin and cytokinin affect the regeneration efficiency of explants (Skoog and Miller, 1957). Kumari et al. found that Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2 mg/L 6-BA and 0.2 mg/L IAA was optimum for shoot regeneration in *Bacopa monniera* (Kumari et al., 2015). In groundnut, a high regeneration efficiency was achieved by adding 66.6  $\mu$ M 6-BA in the medium, while the highest number of shoot buds per explant was achieved by adding 20  $\mu$ M 6-BA and 10  $\mu$ M 2,4-D (Tiwari et al., 2015). The highest regeneration efficiency for cotyledonary nodes of Crambe was observed on basic medium with 0.5  $\mu$ M NAA and 2.2  $\mu$ M 6-BA (Qi et al., 2014).

Gibberellin (GA) is one of the most important hormones affecting plant growth and development (Hua and Irving, 2011). It not only promotes seed germination, hypocotyl elongation, xylem development, and internode elongation, but also induces the differentiation of flower buds (Yukika et al., 1997; Almqvist, 2003; Thomas and Sun, 2004). GA is necessary for stem elongation (Nishijima et al., 1997). A previous study showed that the highest number of shoots per explant and shoot elongation rate was obtained by using 3 mg/L 6-BA in combination with 0.5

mg/L  $GA_3$  during shoot induction stage in tea (Gonbad et al., 2014). MS medium supplemented with 1 mg/L 6-BA, 0.1 mg/L IBA, and 2 mg/L  $GA_3$  promoted shoot elongation significantly in *Cerasus campanulata* (Wang and Huang, 2002). In sweet potato, the combination of 10 mg/L  $GA_3$  and 1 mg/L 6-BA could give rise to significantly taller shoots (20 mm) compared to the rest of the treatments (Masekesa et al., 2016).

Callus browning is another problem to solve for *Agrobacterium*-mediated transformation. Explants will have defense response when infected by *Agrobacterium*. Then the protective layer is formed on the cell surface that results in browning or necrosis. In order to mitigate tissue browning in *Agrobacterium*-mediated transformation, researchers have concentrated on the antioxidants such as ascorbate (Vc),  $\alpha$ -lipoic acid,  $\alpha$ -tocopherol, dithiothreitol (DTT), glutathione, L-cysteine (L-cys), polyvinylpyrrolidone (PVPP), and selenite (Dan, 2008). These antioxidants can reduce tissue browning of transformed cells and improve regeneration efficiency, thus enhancing transformation efficiency. A combination of Vc, silver nitrate ( $AgNO_3$ ) and cysteine improved *Agrobacterium*-mediated transformation efficiency in sugar cane (Enríquez Obregón et al., 1998). Adding Vc to tissue culture medium can significantly reduce the degree of browning, and adding  $AgNO_3$  can not only reduce tissue browning effectively, but also improve the number of adventitious buds significantly in *Zizyphus jujube* (Huang et al., 2006). When  $AgNO_3$  and 6-BA were added in the culture medium during soybean transformation using hypocotyl as explants, the induction rate of adventitious buds was improved (five times higher than the control group) and the rate of hypocotyl browning was reduced (Wang and Xu, 2008). Adding  $\alpha$ -lipoic acid significantly increased the induction rate of transgenic shoots in four crop species including soybean, wheat, tomato, and cotton (Dan et al., 2009). DTT has been found to play positive roles in plant transformation. In soybean, different concentrations of DTT and L-cys were added in the *Agrobacterium* re-suspension medium and CCM to inhibit the necrosis of explants (Olhoft and Somers, 2001; Li et al., 2008b; Zhang et al., 2016). When 0.1% DTT was added in CCM, the regeneration efficiency increased from 8 to 22% in wheat (Yu et al., 2005).

In this study, we tried to improve the *Agrobacterium*-mediated transformation efficiency in soybean by improving the infection efficiency of *Agrobacterium* and regeneration efficiency of explants (shoot elongation rate). The *Agrobacterium* infection efficiencies were compared using different concentrations of *Agrobacterium* for infection, explants, *Agrobacterium* suspension medium, co-cultivation time, and soybean varieties. The rates of shoot elongation were evaluated by adding different concentration combinations of  $GA_3$  and IAA, as well as  $AgNO_3$  or Vc in the tissue culture medium. The *Agrobacterium* infection efficiency was estimated by the rate of GUS transient expression in soybean cotyledonary explants, and the rate of shoot elongation was investigated by the frequencies of elongated shoots. Finally, the transformation efficiency was calculated based on the number of positive transgenic soybean seedlings detected by different methods. The optimized protocol for *Agrobacterium*-mediated transformation in this study would be helpful to further

study gene functions in soybean and develop new varieties by molecular breeding.

## MATERIALS AND METHODS

### Experimental Design

As the overall transformation efficiency is largely determined by the efficiencies of *Agrobacterium* infection and explant regeneration (where shoot elongation rate is the bottleneck in soybean), we investigated the key factors affecting *Agrobacterium* infection efficiency including *Agrobacterium* concentrations, explants, *Agrobacterium* suspension medium, co-cultivation time, and soybean varieties (**Figure 1**, textboxes with red borders), and the major factors affecting the explant regeneration efficiency (shoot elongation rate) including different concentration combinations of GA<sub>3</sub> and IAA in SEM as well as adding AgNO<sub>3</sub> or Vc in SIM and SEM (**Figure 1**, textboxes with blue borders).

### Plant Materials

Soybean varieties including Tianlong 1, Jack Purple, DLH, NN419, Williams 82, HZM, NN34, NN88-1 were used in this study. The seeds were provided by the National Center for Soybean Improvement at Nanjing Agricultural University, Nanjing, China.

### Agrobacterium Strain and Vector

The *A. tumefaciens* strain EHA101 (Hood et al., 1986) and the binary plasmid pTF102 (Frame and Kan, 2002) were used in this study, which were kindly provided by Dr. Huixia Shou (Zhejiang University, China) and Dr. Kan Wang (Iowa State University, USA), respectively. The pTF102 vector contains a *GUS* gene (with intron) as the reporter gene, a *bar* gene as the selectable marker gene (conferring resistance to herbicide glufosinate), and an *aadA* gene for resistance to antibiotics spectinomycin.

### Agrobacterium Culture and Infection Medium

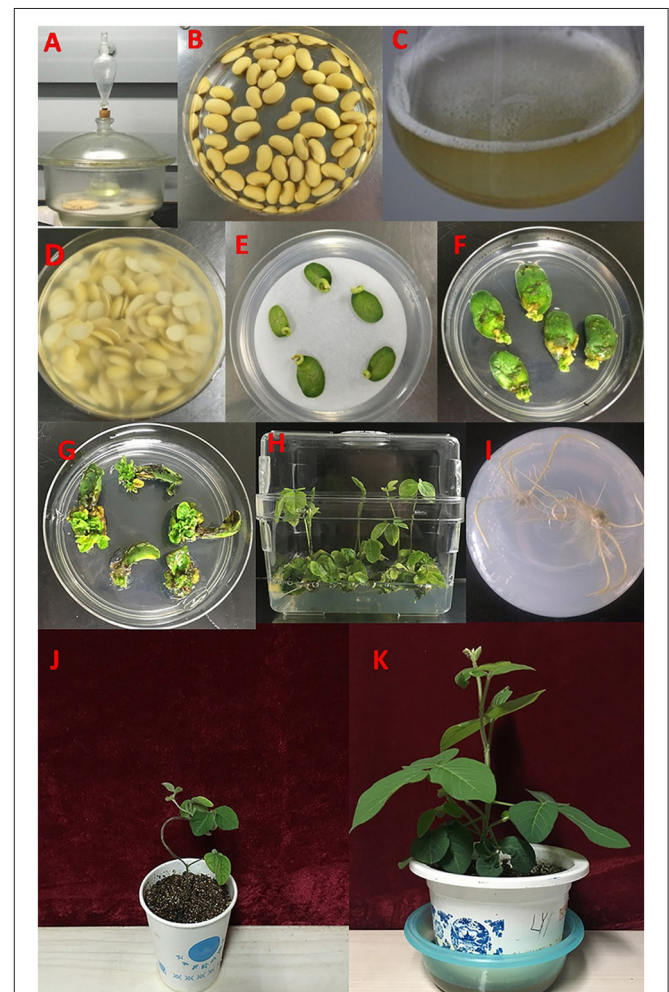
To prepare the *Agrobacterium* infection medium, a single colony of *A. tumefaciens* strain EHA101/pTF102 was picked from the plate and put into 5 ml liquid YEB (An et al., 1989, 5 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract) containing 25 mg/L chloramphenicol, 50 mg/L kanamycin, 50 mg/L streptomycin, and 100 mg/L spectinomycin, and cultured for 24 h at 28°C (250 rpm) to get the starter culture. Next, 200 µl of the starter culture was transferred to 200 ml YEB culture and grown at 28°C (250 rpm) in a shaker incubator until OD<sub>650</sub> reached 0.6, 0.8, or 1.0. The *Agrobacterium* culture was collected and centrifuged at 5000 rpm (22°C) for 10 min, and then the pellet was re-suspended using the liquid CCM until OD<sub>650</sub> reached 0.7. The re-suspended infection medium was shaken for 0.5 h (70 rpm, 22°C) before use. The liquid CCM contains 1/10X B<sub>5</sub> basal medium (Gamborg and Al, 1968) supplemented with 3.9 g/L 2-[N-morpholino] ethanesulfonic acid (MES), 3% (30 g/L) sucrose, pH = 5.4 (adjusting by KOH), 1.67 mg/L 6-BA, 0.25 mg/L GA<sub>3</sub>, 40 mg/L acetosyringone (AS), and DTT (0 or 154.2 mg/L).

## The Process of Soybean Transformation

The protocol for *Agrobacterium*-mediated soybean transformation in this study was based on a previous study (Paz et al., 2006) with modification, which is briefly summarized as the following steps (**Figure 2**).

### Explant Preparation, Agrobacterium Infection and Co-Cultivation

The mature soybean seeds without any defects were wiped clean using a cotton cloth (wetted by 75% alcohol), and then surface-sterilized for 6 or 12 h with chlorine gas (100 ml NaClO + 10 ml HCl or 100 ml NaClO + 3.5 ml HCl) in a tightly sealed chamber (**Figure 2A**). To get the half-seed cotyledonary explants, the sterilized seeds were soaked in sterile water at 23°C under dark for 16–18 h (**Figure 2B**). Then the imbibed seeds were longitudinally cut along the hilum using a scalpel, and the



**FIGURE 2 |** The experimental process of *Agrobacterium*-mediated transformation using half-seed soybean explants. **(A)** Seed sterilization. **(B)** Seed imbibition. **(C)** Preparation of *Agrobacterium*. **(D)** *Agrobacterium* infection of half-seed cotyledonary explants. **(E)** Co-cultivation. **(F, G)** Shoot induction. **(H)** Shoot elongation. **(I)** Rooting. **(J, K)** Transplanting and adaptation to normal growth condition.

seed coats were removed. The hypocotyls were trimmed to 3 mm. The half-seed cotyledonary explants or cotyledonary nodes from 1-, 3-, or 5-d-old seedlings were put in the prepared re-suspended *Agrobacterium* infection medium (Figure 2C) for 30 min (Figure 2D). The *Agrobacterium* suspension medium was shaken during the period of infection to make the explants in well contact with the *Agrobacterium* liquid. After infection, five to seven explants (adaxial side up) were evenly placed on sterile filter paper over solid CCM in Petri dishes (90 mm in diameter × 15 mm deep), and co-cultivated at 23°C under a photoperiod of 16 h/8 h (light/dark) for 3–5 d (Figure 2E). The solid CCM contains 1/10X B<sub>5</sub> basal medium supplemented with 3.9 g/L MES, 3% (30 g/L) sucrose, 0.5% (5 g/L) agrose (Biowest, Spain), pH = 5.4 (adjusting by KOH), 1.67 mg/L 6-BA, 0.25 mg/L GA<sub>3</sub>, 40 mg/L AS, 400 mg/L L-cys, 154.2 mg/L DTT, and 158 mg/L sodium thiosulfate.

### Tissue Culture and Transplanting

After co-cultivation, the explants were inserted (tilting 45 degrees) in SIM with the adaxial side facing upward, and maintained in a walk-in chamber under the photoperiod of 16 h/8 h (light/dark) and temperature of 23°C (Figures 2F,G). The SIM contains B<sub>5</sub> basal medium supplemented with 3% sucrose, 0.35% (3.5 g/L) phytigel (Sigma, USA), 0.58 g/L MES, pH 5.6, 1.67 mg/L 6-BA, 50 mg/L cefotaxime (Cef), 500 mg/L carbenicillin (Carb), and 5 mg/L glufosinate (Sigma, USA). Two weeks later, the explants were transferred to fresh SIM. Four weeks after shoot induction, the explants were transferred to SEM, which contains MS basal medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.35% (3.5 g/L) phytigel (Sigma, USA), 0.58 g/L MES, pH 5.6, 0.5 mg/L GA<sub>3</sub>, 0.1 mg/L IAA, 1 mg/L zeatin (ZR), 50 mg/L asparagine (Asp), 100 mg/L L-pyrogutamic acid (L-pyro), 75 mg/L Cef, 500 mg/L Carb, and 3 mg/L glufosinate. The explants were transferred to fresh SEM every two weeks until the elongated shoots reached 3 cm high (Figure 2H). Elongated shoots were placed into rooting medium (B<sub>5</sub> basal medium supplemented with 1.5% sucrose, 0.8% agar powder, 0.59 g/L MES, pH 5.7, and 1 mg/L IBA), until the roots were developed to 2–3 cm in length (Figure 2I). Eventually the plants were transplanted in pots (soil: vermiculite = 1:1) and grown in greenhouse at 28/24°C with a photoperiod of 16 h/8 h (light/dark) until maturity (Figures 2J,K).

### Identification of Positive Transgenic Soybean Plants

We used the following four methods to detect the positive transgenic soybean plants.

#### GUS Histochemical Staining

The young leaves of transgenic soybean plants were collected and submerged in GUS staining solution (Table S1) overnight in dark at 37°C, then rinsed in 75% alcohol two or three times to remove chlorophyll (Jefferson et al., 1987). The tissues of positive transgenic plants would show blue color.

#### Herbicide (glufosinate) Painting

Half (along the midrib) of a leaf (the upper surface) was painted with 135 mg/L glufosinate using a swab, while a black line was

drawn on the other half leaf to mark it as control. About seven days later, if the half leaf with glufosinate treatment is same as the control, the plant is tolerant to herbicide and therefore positive.

#### PCR Assay

Total genomic DNA was extracted using the CTAB method (Paterson et al., 1993). Gene specific primers were designed by NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), and were used to amplify the 1812 bp *GUS* fragment and 428 bp *bar* fragment (Table S2). The PCR condition was set as the following: one cycle at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 1 min 50 s (*GUS*), or 30 s (*bar*), and a final extension at 72°C for 5 min. The amplified PCR products were visualized and photographed using the gel imaging system after electrophoresis on a 0.8% (w/v) agarose gel containing 120 ul/L ethidium bromide.

#### LibertyLink® Strip (*bar*) Test

Leaf tissue was put in an Eppendorf tube and grounded with 0.5 ml extraction buffer by a pestle. Then a LibertyLink® strip was inserted into the tube and waited for 10 min. The appearance of a control line indicates the strip is functional and the second line (test line) indicates the sample is positive.

#### Analysis of Gene Copy Number by Absolute Quantification PCR

The copy number of *bar* gene integrated in the soybean genomic DNA was estimated by the ratio of exogenous target gene (*bar*)/internal reference gene (*lectin*),  $X_0/R_0$ , which could be calculated according to the formula:  $X_0/R_0 = 10^{[(Ct,X-IX)/SX]-[(Ct,R-IR)/SR]}$  (Weng et al., 2004), where  $Ct_{t,X}$  and  $Ct_{t,R}$  represents the Ct value of target gene (*bar*) and reference gene (*lectin*), respectively; IX and SX is the intercept and slope of the standard curve of the target gene, respectively; IR and SR is the intercept and slope of the standard curve of the reference gene, respectively. The stand curve was obtained by plotting the logarithms of the template DNA copy number (X axis) against the Ct values (Y axis) using a series of DNA template dilutions, where the template DNA copy number = avogadro constant ( $6.02 \times 10^{23}$ ) × concentration of template DNA (g/ml)/the relative molecular mass of template DNA (g/mol), and the Ct values were obtained by absolute quantification PCR. The standard curves of the exogenous *bar* gene (on the positive plasmid) and the internal *lectin* gene (on the genomic DNA) were established respectively (Sambrook and Russell, 2001). The size of positive plasmid we used in this study is 11,622 bp, and the genome size of soybean is 1,150 Mb (Schmutz et al., 2010). Because two soybean varieties were used for transformation, the series dilutions of genomic DNA from the control plants of both Jack Purple and Tianlong 1, as well as the positive plasmid were made respectively to generate the standard curves.

Quantification PCR was performed on the LightCycler 480 (Roche, USA) to obtain the Ct values of the exogenous target *bar* gene and the internal reference *lectin* gene (Qiu et al., 2012). The 20 μl qRT-PCR reaction mixture contained the following components: 1 μl (100 ng) template DNA, 10 μl 2 × SYBR® Premix Taq™, 0.4 μl (20 μM) of the forward and reverse primers

(Table S2), and 8.2  $\mu$ l of sterile ddH<sub>2</sub>O. The PCR condition was set as one cycle at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 15 s. Each sample was repeated three times.

### Determination of *Agrobacterium* Infection Efficiency, Rate of Shoot Elongation and transformation Efficiency

The *Agrobacterium* infection efficiency was estimated by the rate of GUS transient expression in soybean cotyledonary explants. The rate of GUS transient expression (%) = (The number of cotyledonary explants in blue color/total number of cotyledonary explants for staining)  $\times$  100%. The rate of shoot elongation (%) = (The number of elongated shoots with heights  $\geq$  3 cm/the number of infected explants)  $\times$  100%. The transformation efficiency (%) = (The number of positive transgenic plants / the number of infected explants)  $\times$  100%.

### Statistical Analysis

SAS 9.2 software was used for statistical analysis. Differences between two groups were analyzed by student's *t*-test, while differences among multiple treatments were analyzed using Duncan's multiple range test. GraphPad Prism, Microsoft Excel, and PowerPoint were used to generate graphs.

## RESULTS

### The Effect of DTT on the Transient GUS Expression in Soybean Explants

The rate of GUS transient expression in the cotyledonary explants reflects the infection efficiency of *Agrobacterium*. In order to see the effect of the antioxidant DTT on the transient GUS expression in explants, 0 or 154.2 mg/L DTT was added to the re-suspended infection medium after collecting the *Agrobacterium* when OD<sub>650</sub> = 0.6. The half-seed explants from 1-d imbibition were infected by the *Agrobacterium* suspension medium with or without DTT, and subjected to GUS staining after 5 days of co-cultivation. According to the GUS staining intensity, the explants were divided into three categories: strong, weak, and none (Figure 3). The rate of total transient GUS expression in the cotyledonary explants after adding DTT in re-suspended

infection medium was increased by 13 and 3% for Jack Purple and Tianlong 1, respectively (Figures 4A,B), compared with the control (without DTT), and the rate of strong GUS transient expression was significantly ( $P < 0.05$ , student's *t*-test) increased by 16 and 35% for Jack Purple and Tianlong 1, respectively (Figures 4C,D).

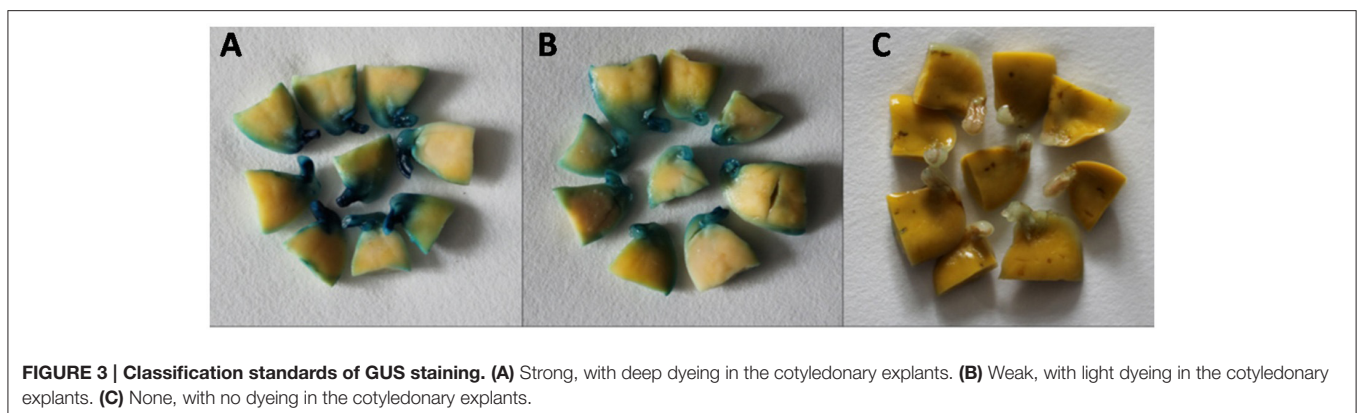
### The Effect of *Agrobacterium* Concentration on the Transient GUS Expression in Explants

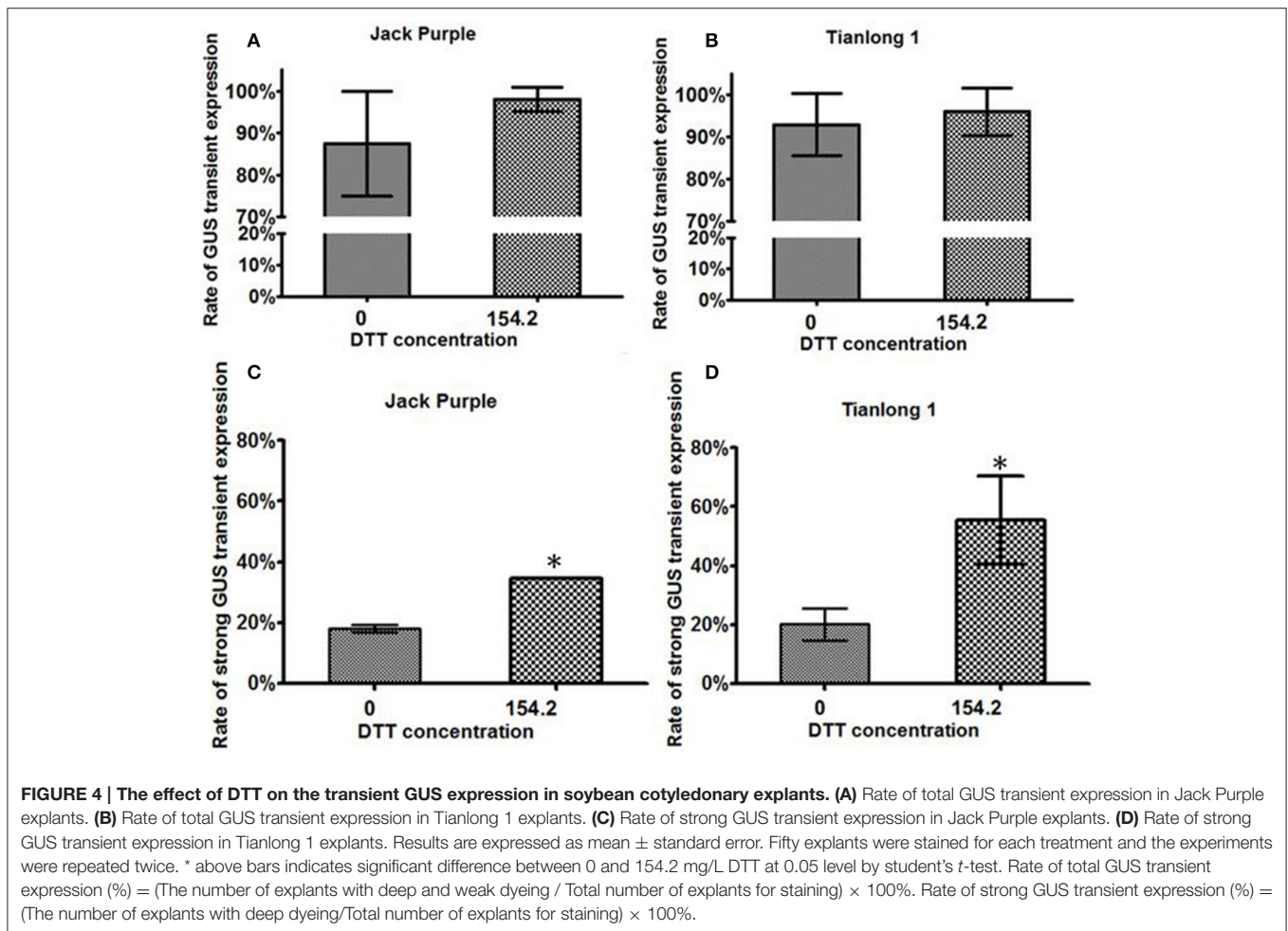
To determine the optimal concentration of *Agrobacterium* for infection, the *Agrobacterium* at three different OD<sub>650</sub> (0.6, 0.8, 1.0) during logarithmic growth stage (Figure S1) were collected, respectively, and re-suspended in the liquid CCM containing 154.2 mg/L DTT, then co-cultured with soybean half-seed explants (from 1-d imbibition) for 5 days. The explants were stained after 5 days of co-cultivation.

The rate of total GUS transient expression in the cotyledonary explants of both soybean varieties (Jack Purple and Tianlong 1) was not significantly different among the concentrations of OD<sub>650</sub> = 0.6, 0.8, and 1.0 (Figures 5A,B). However, for both soybean varieties, the rate of strong GUS transient expression was significantly ( $P < 0.05$ , Duncan's multiple range test) higher when using the concentration of OD<sub>650</sub> = 0.6 than OD<sub>650</sub> = 1, and slightly higher than OD<sub>650</sub> = 0.8 (Figures 5C,D). Therefore, we conclude that the optimal concentration of *Agrobacterium* for infection is OD<sub>650</sub> = 0.6.

### The Effect of Different Explants on the Transient GUS Expression

The *Agrobacterium* infection efficiencies were then compared among four different explants prepared by 1-d imbibition, 1-d germination, 3-d germination, and 5-d germination, respectively, using two soybean varieties of Jack Purple and Tianlong 1. The *Agrobacterium* was collected at OD<sub>650</sub> = 0.6, and re-suspended in the liquid CCM containing 154.2 mg/L DTT to infect above four different explants, respectively. After 5 days of co-cultivation, the explants were subjected to GUS staining. The result showed that the highest rate of total GUS transient expression in the cotyledonary explants was achieved when using the half-seed explants from 1-d imbibition for both soybean varieties, 95 and





97% in Jack Purple and Tianlong 1, respectively, which was significantly ( $P < 0.05$ , Duncan's multiple range test) higher than the other three types of explants (Figure 6).

### The Effect of Co-Cultivation Time on Transient GUS Expression in Soybean Explants

The *Agrobacterium* was collected at  $OD_{650} = 0.6$ , and re-suspended in the liquid CCM containing 154.2 mg/L DTT to infect the soybean half-seed explants from 1-d imbibition. After 3, 4, 5, or 6 days of co-cultivation, the explants were subjected to GUS staining. The results showed that the transient GUS expression rates after four different co-cultivation time were not significantly different (Figure 7). However, the explants were almost yellow when co-cultivated for 3 or 4 days but turned green after 5 days (Figure S2). Therefore, 5-d co-cultivation is chosen when the explants have a strong vitality.

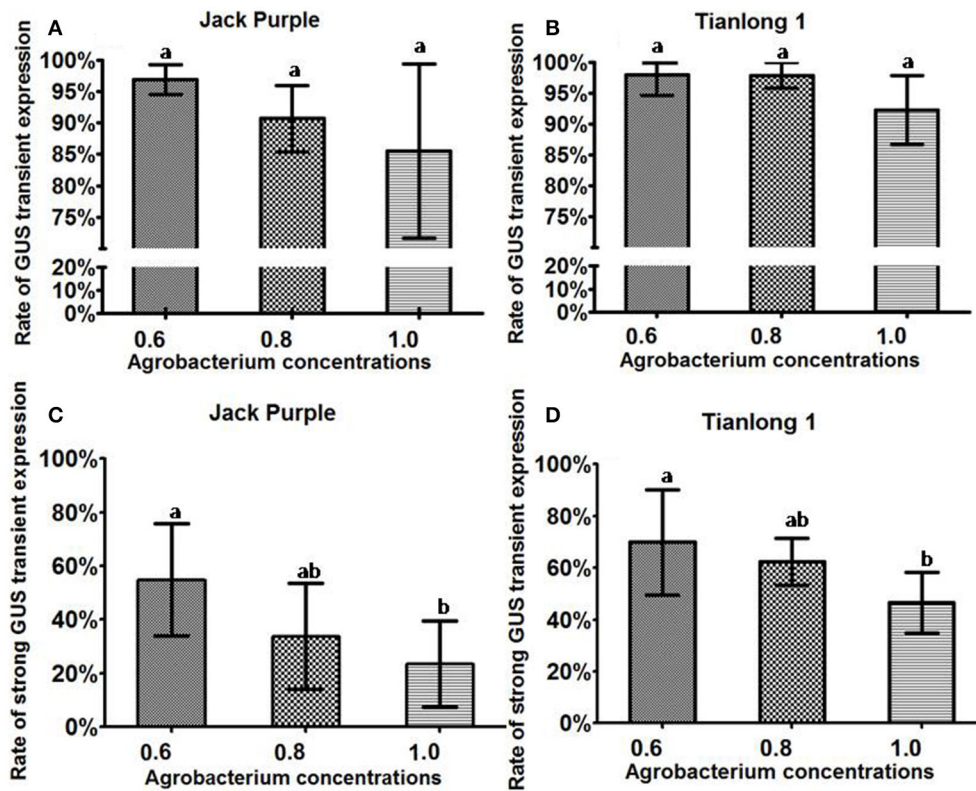
### Rate of Transient GUS Expression in Different Soybean Varieties

Eight soybean varieties, including Tianlong 1, Jack Purple, DLH, NN419, Williams 82, HZM, NN34, and NN88-1, were used

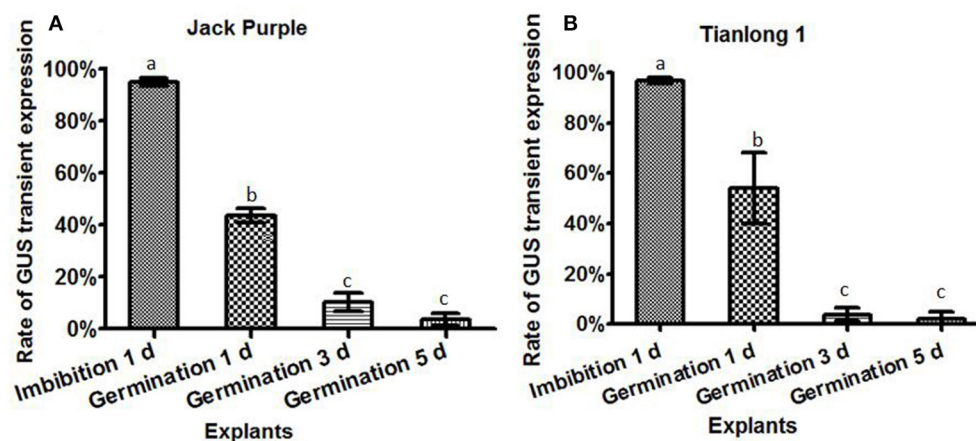
to compare the soybean genotype effect on the *Agrobacterium* infection efficiency, with all the other factors optimized (which was collecting the *Agrobacterium* when  $OD_{650} = 0.6$ , then re-suspended in the liquid CCM with 154.2 mg/L DTT to infect the half-seed explants from 1-d imbibition, and co-cultured for 5 days). The results showed that the rate of transient GUS expression in Tianlong 1 and Jack Purple was significantly ( $P < 0.05$ , Duncan's multiple range test) higher than in HZM, NN34, and NN88-1 (Figure 8). We chose Tianlong 1 and Jack Purple in further experiments because of their higher rate of transient GUS expression.

### The Effect of Different Concentration Combinations of GA<sub>3</sub> and IAA on the Rate of Shoot Elongation and Transformation Efficiency in Soybean

GA<sub>3</sub> and IAA are important phytohormones to regulate plant growth. GA<sub>3</sub> promotes stem elongation while IAA mainly promotes cell elongation (Ji and Yang, 2002). Optimum phytohormone concentration is a key factor to improve the regeneration efficiency and transformation efficiency of plants. Based on the original concentration of GA<sub>3</sub> and IAA (0.5 mg/L

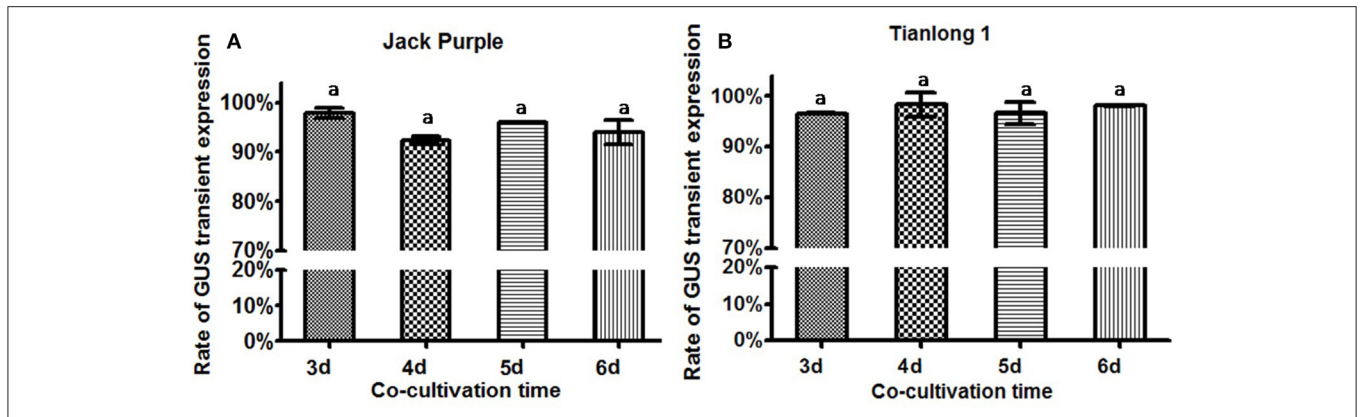


**FIGURE 5 | The effect of *Agrobacterium* concentration on the transient GUS expression in soybean cotyledonary explants. (A)** Rate of total GUS transient expression in the cotyledonary explants of Jack Purple. **(B)** Rate of total GUS transient expression in the cotyledonary explants of Tianlong 1. **(C)** Rate of strong GUS transient expression in the cotyledonary explants of Jack Purple. **(D)** Rate of strong GUS transient expression in the cotyledonary explants of Tianlong 1. Results are expressed as mean  $\pm$  standard error. Fifty explants were stained for each treatment and the experiments were repeated five times. Means with the same letter above bars are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of total GUS transient expression (%) = (The number of explants with deep and weak dyeing/Total number of explants for staining)  $\times$  100%. Rate of strong GUS transient expression (%) = (The number of explants with deep dyeing/Total number of explants for staining)  $\times$  100%.

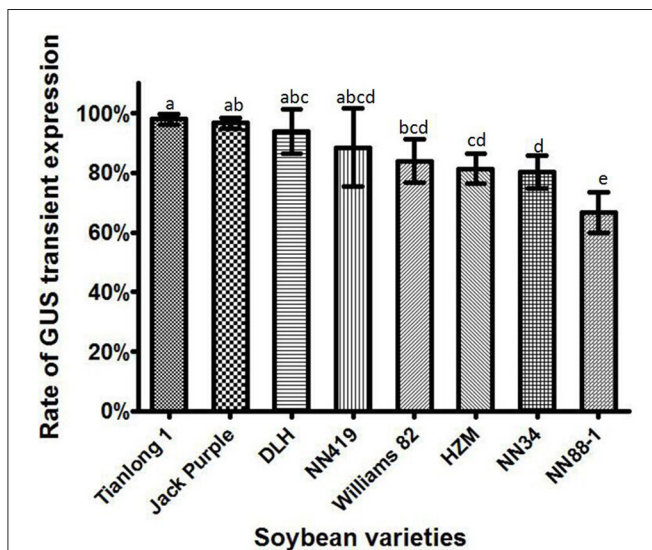


**FIGURE 6 | The effect of different explants on the transient GUS expression.** The cotyledonary explants were obtained by four different methods, including imbibition for 1 d, germination for 1, 3, and 5 d. **(A)** Rate of total GUS transient expression in the cotyledonary explants of Jack Purple. **(B)** Rate of total GUS transient expression in the cotyledonary explants of Tianlong 1. The results are expressed as mean  $\pm$  standard error. Fifty explants were stained for each treatment and the experiments were repeated twice. Means with the same letter above bars are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of total GUS transient expression (%) = (The number of explants with deep and weak dyeing/Total number of explants for staining)  $\times$  100%.





**FIGURE 7 | The effect of co-cultivation time on transient GUS expression in soybean explants. (A)** Rate of total GUS transient expression in the cotyledonary explants of Jack Purple. **(B)** Rate of total GUS transient expression in the cotyledonary explants of Tianlong 1. Results are expressed as mean  $\pm$  standard error. Fifty explants were stained for each treatment and the experiments were repeated twice. Means with the same letter above bars are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of total GUS transient expression (%) = (The number of explants with deep and weak dyeing/Total number of explants for staining)  $\times$  100%.



**FIGURE 8 | The transient GUS expression in the cotyledonary explants of different soybean varieties.** Results are expressed as mean  $\pm$  standard error. Fifty explants were stained for each soybean variety in every replication and the experiments were repeated three times. Means with the same letter above bars are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of total GUS transient expression (%) = (The number of explants with deep and weak dyeing/Total number of explants for staining)  $\times$  100%.

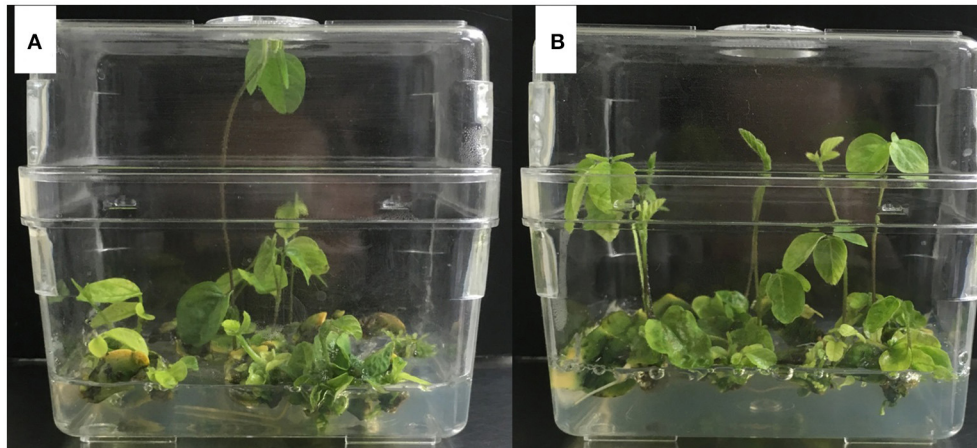
**TABLE 1 | The effect of combination of different GA<sub>3</sub> and IAA concentrations on the rate of shoot elongation and transformation efficiency in soybean.**

Soybean variety	GA <sub>3</sub> concentration (mg/L)	IAA concentration (mg/L)	Rate of shoot elongation (%)	Transformation efficiency (%)
Jack Purple	0.5	0.1	16.11 $\pm$ 0.05 <sup>b</sup>	5.00 $\pm$ 0.03 <sup>a</sup>
	0.5	0.2	13.70 $\pm$ 0.02 <sup>b</sup>	4.57 $\pm$ 0.01 <sup>a</sup>
	1.0	0.1	33.54 $\pm$ 0.03 <sup>a</sup>	7.32 $\pm$ 0.01 <sup>a</sup>
	1.0	0.2	17.42 $\pm$ 0.01 <sup>b</sup>	5.26 $\pm$ 0.01 <sup>a</sup>
	1.5	0.1	11.49 $\pm$ 0.01 <sup>b</sup>	5.41 $\pm$ 0.03 <sup>a</sup>
	1.5	0.2	13.39 $\pm$ 0.05 <sup>b</sup>	5.36 $\pm$ 0.01 <sup>a</sup>
Tianlong 1	0.5	0.1	14.75 $\pm$ 0.01 <sup>ab</sup>	4.28 $\pm$ 0.00 <sup>a</sup>
	0.5	0.2	10.60 $\pm$ 0.01 <sup>b</sup>	1.76 $\pm$ 0.02 <sup>a</sup>
	1.0	0.1	26.08 $\pm$ 0.07 <sup>a</sup>	10.01 $\pm$ 0.03 <sup>a</sup>
	1.0	0.2	9.80 $\pm$ 0.04 <sup>b</sup>	3.53 $\pm$ 0.02 <sup>a</sup>
	1.5	0.1	19.56 $\pm$ 0.05 <sup>ab</sup>	4.49 $\pm$ 0.03 <sup>a</sup>
	1.5	0.2	14.40 $\pm$ 0.03 <sup>ab</sup>	5.69 $\pm$ 0.04 <sup>a</sup>

The results are expressed as mean  $\pm$  standard error. Eighty explants were infected by *Agrobacterium* for each combination/treatment and the experiments were repeated twice. The concentrations of glufosinate for selection were 5 mg/L and 3 mg/L in SIM and SEM, respectively. The numbers of elongated shoots (height  $\geq$  3 cm) were recorded during SEM stage. For each soybean variety, means with the same letter are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of shoot elongation (%) = (The number of elongated shoots/the number of infected explants)  $\times$  100%. Transformation efficiency (%) = (The number of the positive plants/the number of infected explants)  $\times$  100%.

GA<sub>3</sub> and 0.1 mg/L IAA) in SEM (Paz et al., 2006), we used three levels of GA<sub>3</sub> concentration (0.5, 1.0, and 1.5 mg/L) and two levels of IAA concentration (0.1 and 0.2 mg/L) in the experimental design, which would be six combinations (Table 1). The rate of shoot elongation and transformation efficiency in Jack Purple and Tianlong 1 were calculated respectively (Table 1). The results showed that the highest rate of shoot elongation in Jack Purple was 33.54% when 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA

were added in SEM, which was significantly ( $P < 0.05$ , Duncan's multiple range test) higher than the original concentration combination of GA<sub>3</sub> and IAA (0.5 mg/L GA<sub>3</sub> and 0.1 mg/L IAA) in SEM (Table 1, Figure 9). Similar results were found for Tianlong 1. The highest rate of shoot elongation in Tianlong 1 was 26.08% when 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA were added in SEM (Table 1). The transformation efficiencies in Jack Purple



**FIGURE 9 |** The explants of soybean variety Jack Purple in the shoot elongation medium. (A) Soybean explants on SEM with 0.5 mg/L GA<sub>3</sub> and 0.1 mg/L IAA. (B) Soybean explants on SEM with 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA.

and Tianlong 1 were 7.32 and 10.01% when SEM contained 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA, which were higher than 5.00 and 4.28% when SEM contained 0.5 mg/L GA<sub>3</sub> and 0.1 mg/L IAA, respectively (Table 1). These results suggest that the optimal concentration combination of GA<sub>3</sub> and IAA (1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA) improved the rate of shoot elongation and transformation efficiency in soybean varieties of Jack Purple and Tianlong 1.

### The Effect of AgNO<sub>3</sub> and Vc on the Rate of Shoot Elongation and Transformation Efficiency in Soybean

Tissue browning is another factor affecting transformation efficiency. 88-1 is one of the soybean varieties that encountered more serious tissue browning. Therefore, we added AgNO<sub>3</sub> (5, 10, or 15 mg/L) to SIM and SEM (with 0.5 mg/L GA<sub>3</sub> and 0.1 mg/L IAA in SEM) during tissue culturing 88-1 explants. We did not observe alleviation of the tissue browning after adding AgNO<sub>3</sub>. However, the shoot elongation rate increased after adding different concentrations of AgNO<sub>3</sub>, and the transformation efficiency in 88-1 improved from 3.17 to 5.50% after adding 15 mg/L AgNO<sub>3</sub> (Table S3).

Therefore, we further investigated the effect of adding 15 mg/L AgNO<sub>3</sub> in SIM and SEM (with 0.1 mg/L IAA in SEM) using the other two soybean varieties of Jack Purple and Tianlong 1. Because doubling GA<sub>3</sub> concentration in SEM improved the rate of shoot elongation (Table 1), we investigated the effect of AgNO<sub>3</sub> under two levels of GA<sub>3</sub> concentration in SEM. In Jack Purple, the results showed that the rate of shoot elongation and transformation efficiency decreased when adding 15 mg/L AgNO<sub>3</sub> in SIM and SEM (Table 2), and abnormal leaves were observed on Jack Purple after adding 15 mg/L AgNO<sub>3</sub> in the medium (Figure S3). In Tianlong 1, the rate of shoot elongation increased from 14.75 to 19.47% at the level of 0.5 mg/L GA<sub>3</sub> and from 26.08 to 31.82% at the level of 1.0 mg/L GA<sub>3</sub>. However, adding 15 mg/L AgNO<sub>3</sub> in SIM

**TABLE 2 |** The effect of AgNO<sub>3</sub> on the rate of shoot elongation and transformation efficiency in soybean.

Soybean variety	GA <sub>3</sub> concentration (mg/L)	AgNO <sub>3</sub> concentration (mg/L)	Rate of shoot elongation (%)	Transformation efficiency (%)
Jack Purple	0.5	0	16.11 ± 0.05 <sup>b</sup>	5.00 ± 0.03 <sup>a</sup>
	0.5	15	14.44 ± 0.00 <sup>b</sup>	3.89 ± 0.02 <sup>a</sup>
	1	0	33.54 ± 0.03 <sup>a</sup>	7.32 ± 0.01 <sup>a</sup>
	1	15	26.22 ± 0.02 <sup>ab</sup>	6.71 ± 0.01 <sup>a</sup>
Tianlong 1	0.5	0	14.75 ± 0.01 <sup>a</sup>	4.28 ± 0.00 <sup>a</sup>
	0.5	15	19.47 ± 0.03 <sup>a</sup>	5.44 ± 0.03 <sup>a</sup>
	1	0	26.08 ± 0.07 <sup>a</sup>	10.01 ± 0.03 <sup>a</sup>
	1	15	31.82 ± 0.00 <sup>a</sup>	9.09 ± 0.01 <sup>a</sup>

The results are expressed as mean ± standard error. Eighty explants were infected by *Agrobacterium* for each combination/treatment and the experiments were repeated twice. The concentrations of glufosinate for selection were 5 mg/L and 3 mg/L in SIM and SEM, respectively. The numbers of elongated shoots (height ≥ 3 cm) were recorded during SEM stage. For each soybean variety, means with the same letter are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of shoot elongation (%) = (The number of elongated shoots/the number of infected explants) × 100%. Transformation efficiency (%) = (The number of the positive plants / the number of infected explants) × 100%.

and SEM had little effect on the transformation efficiency (Table 2).

Previous studies showed that adding 200 mg/L Vc in the medium can prevent tissue browning in *Bromeliaceae* (Peng et al., 2007) and promote rooting in *Limonium* (Xu et al., 2010). Therefore, we added 200 mg/L Vc to SIM and SEM (with 0.1 mg/L IAA in SEM) to see if Vc can prevent browning and improve the rate of shoot elongation and transformation efficiency in Jack Purple and Tianlong 1. For Jack Purple, the rate of shoot elongation increased from 16.11 to 23.17%, and the transformation efficiency increased from 5.00 to 9.15% after adding 200 mg/L Vc in SIM and SEM when GA<sub>3</sub> concentration

was 0.5 mg/L in SEM. When the GA<sub>3</sub> concentration was 1 mg/L in SEM, the rate of shoot elongation was reduced but the transformation efficiency increased slightly (Table 3). For Tianlong 1, the rate of shoot elongation and transformation efficiency was slightly improved after adding 200 mg/L Vc in SIM and SEM when GA<sub>3</sub> concentration was 0.5 mg/L in SEM. However, at 1 mg/L GA<sub>3</sub> in SEM, the rate of shoot elongation and transformation efficiency decreased after adding 200 mg/L Vc in SIM and SEM. These results suggested that the tissue culture medium should be optimized specifically for different soybean varieties.

## Confirmation of Positive Transgenic Soybean Plants

The positive T<sub>0</sub> transgenic soybean plants were first identified by GUS staining of young leaves during rooting stage. A total of 20 positive transgenic plants were randomly picked for further confirmation using the other four different methods, including GUS staining after transplanting, PCR amplification of exogenous gene, herbicide (glufosinate) painting, and LibertyLink<sup>®</sup> strip detection. The results (Table 4) showed that all methods were consistent except that GUS staining after transplanting missed four positive transgenic plants. Therefore, GUS staining during rooting stage is recommended first since this method can identify positive transgenic plants as soon as possible, and herbicide painting would be an easy and reliable method to confirm the results.

The positive T<sub>1</sub> transgenic soybean plants were also confirmed by four different methods (Figure 10). The copy number of *bar* gene was analyzed by absolute quantitative PCR using *lectin* as the reference gene (Qiu et al., 2012). The melting curves of *bar* and *lectin* genes showed specific amplification (Figure S4). The

equation for the standard curve of the endogenous reference gene *lectin* in Jack Purple and Tianlong 1 was  $y = -3.483x + 35.33$  ( $R^2 = 0.995$ ), and  $y = -3.167x + 36.49$  ( $R^2 = 0.986$ ), respectively; while the equation for the standard curve of *bar* gene in plasmid was  $y = -3.116x + 40.78$  ( $R^2 = 0.984$ ). Then the ratio of exogenous gene (*bar*) and reference gene (*lectin*) was calculated. Because *lectin* is a single gene in soybean genome (Vodkin et al., 1983), so the ratio of exogenous gene (*bar*) and reference gene (*lectin*) represents the copy of the exogenous gene (*bar*). The results showed that transgenic soybean line 3 and line 6 had one copy, line 2, and line 4 had two copies, line 1 and line 5 had three copies of *bar* gene (Table 5).

## DISCUSSION

Since the cotyledonary nodes were used as explants to obtain the first transgenic soybean plant by *Agrobacterium*-mediated transformation (Hinchee et al., 1988), this method has been modified and an improved transformation efficiency of 3.8% (on average) was achieved (Paz et al., 2006). However, this efficiency is still low to sufficiently screen enough transformation events for molecular breeding or gene function studies. Therefore, we try to further improve the transformation efficiency by increase the *Agrobacterium* infection efficiency and the rate of shoot elongation in soybean.

When the *Agrobacterium* concentration is too high to infect the explants, it is hard to wash the *Agrobacterium* away from the explants and will lead to *Agrobacterium* contamination. But if the concentration of *Agrobacterium* is too low, the infection ability is weak (Zhong, 2007). Therefore, it is better to choose a relatively low concentration with high infection ability. In this study, we found that OD<sub>650</sub> = 0.6 is the optimum concentration for infection. In addition, other factors affecting *Agrobacterium* infection efficiency were also investigated by estimation of the rate of GUS transient expression in soybean cotyledonary explants, including soybean explants, *Agrobacterium* suspension medium, and co-cultivation time. An infection efficiency of over 96% was achieved by collecting the *Agrobacterium* at a concentration of OD<sub>650</sub> = 0.6, then re-suspended in liquid CCM containing 154.2 mg/L DTT to infect the half-seed cotyledonary explants (from mature seeds imbibed for 1 day), and co-cultured them for 5 days. Among the eight soybean varieties, higher *Agrobacterium* infection efficiencies were observed for soybean varieties Tianlong 1, Jack Purple, DLH, and NN419 (Figure 8).

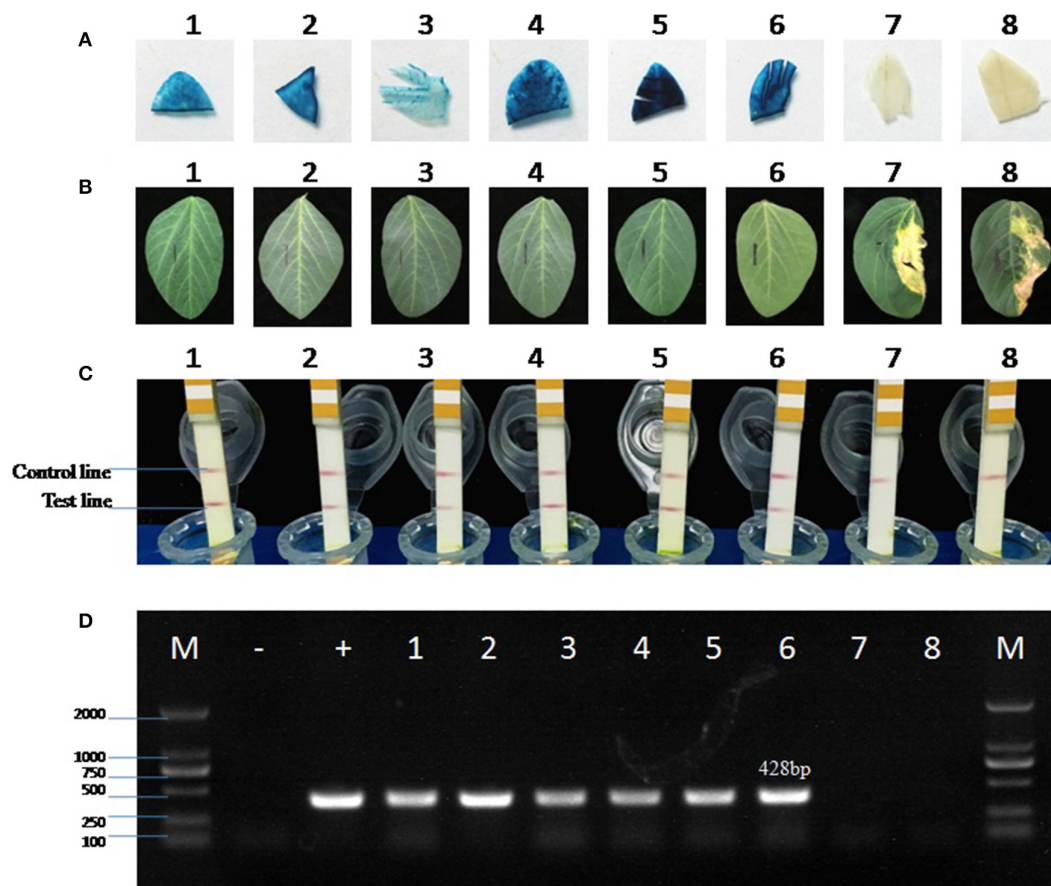
**TABLE 3 | The effect of Vc on the rate of shoot elongation and transformation efficiency in soybean.**

Soybean variety	GA <sub>3</sub> concentration (mg/L)	Vc concentration (mg/L)	Rate of shoot elongation (%)	Transformation efficiency (%)
Jack Purple	0.5	0	16.11 ± 0.05 <sup>b</sup>	5.00 ± 0.03 <sup>a</sup>
	0.5	200	23.17 ± 0.04 <sup>ab</sup>	9.15 ± 0.02 <sup>a</sup>
	1	0	33.54 ± 0.03 <sup>a</sup>	7.32 ± 0.01 <sup>a</sup>
	1	200	28.05 ± 0.04 <sup>ab</sup>	8.54 ± 0.02 <sup>a</sup>
Tianlong 1	0.5	0	14.75 ± 0.01 <sup>a</sup>	4.28 ± 0.00 <sup>a</sup>
	0.5	200	17.73 ± 0.02 <sup>a</sup>	4.55 ± 0.00 <sup>a</sup>
	1	0	26.08 ± 0.07 <sup>a</sup>	10.01 ± 0.03 <sup>a</sup>
	1	200	19.09 ± 0.02 <sup>a</sup>	4.55 ± 0.01 <sup>a</sup>

The results are expressed as mean ± standard error. Eighty explants were infected by *Agrobacterium* for each combination/treatment and the experiments were repeated twice. The concentrations of glufosinate for selection were 5 mg/L and 3 mg/L in SIM and SEM, respectively. The numbers of elongated shoots (height ≥ 3 cm) were recorded during SEM stage. For each soybean variety, means with the same letter are not significantly different at 0.05 level according to Duncan's multiple range test. Rate of shoot elongation (%) = (The number of elongated shoots/the number of infected explants) × 100%. Transformation efficiency (%) = (The number of the positive plants/the number of infected explants) × 100%.

**TABLE 4 | Comparison of different methods to detect positive transgenic soybean plants at T<sub>0</sub> generation.**

Method	Number of positive plants/Total plants	Time after shoot elongation	Time needed for testing
GUS staining during rooting	20/20	1 day	Overnight
GUS staining after transplanting	16/20	2 weeks	Overnight
PCR amplification of GUS gene	20/20	3–4 weeks	2 h
Glufosinate painting on leaves	20/20	4 weeks	7 days
LibertyLink <sup>®</sup> strip detection	20/20	4 weeks	10 min



**FIGURE 10 | Detection of the positive transgenic soybean plants. (A)** GUS staining of soybean leaves. **(B)** Herbicide (glufosinate) painting on soybean leaves. The left was marked by a black line as control, and the right was painted by 135 mg/L glufosinate. **(C)** LibertyLink® strip detection. The first line is control line, and the second line is test line. **(D)** PCR amplification of the 428-bp *bar* gene fragment using negative control (–, ddH<sub>2</sub>O), positive control (+, plasmid pTF102), or soybean genomic DNA as the template. M, 2000 bp DNA marker. 1–3, positive transgenic soybean plants in Jack Purple background. 4–6, positive transgenic soybean plants in Tianlong 1 background. 7, negative control of Jack Purple. 8, negative control of Tianlong 1.

It has been reported in other plant species that the optimum concentration of GA<sub>3</sub> and IAA in SEM could improve the rate of shoot elongation effectively (Gonbad et al., 2014). We investigated six different concentration combinations of GA<sub>3</sub> and IAA in SEM, and the highest rate of shoot elongation was achieved (33.54% in Jack Purple and 26.08% in Tianlong 1) when 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA were added in SEM (Table 1). A previous study showed that a higher elongation rate was observed when adding 0.5 mg/L GA<sub>3</sub> without IAA in SEM when using the soybean variety Heinong 35 explants (Li et al., 2008a). In another study, the highest shoot elongation rate was obtained by adding 1 mg/L GA<sub>3</sub> and 0.5 mg/L IAA to SEM for soybean variety Jiyu47 (Sun, 2013). These results suggest that the optimal concentration combination of GA<sub>3</sub> and IAA in SEM varies among different soybean genotypes.

Plants will generate reactive oxygen species upon the infection of *Agrobacterium*, which leads to cell death and tissue browning. Low concentrations of antioxidants can prevent cell necrosis and improve the transformation efficiency. In this study, the rate of strong GUS transient expression in the cotyledonary

explants of Jack Purple and Tianlong 1 increased by 16 and 35%, respectively, after adding the antioxidant DTT in CCM (Figure 4). The previous study showed that adding Vc in the medium can reduce the degree of tissue browning significantly, and the addition of AgNO<sub>3</sub> can not only reduce tissue browning effectively, but also improve the regeneration efficiency and the number of shoots significantly (Huang et al., 2006). However, in our experiment, the addition of AgNO<sub>3</sub> or Vc in SIM and SEM did not reduce the browning of the explants. Adding 15 mg/L AgNO<sub>3</sub> in SIM and SEM improved the rate of shoot elongation and transformation efficiency in 88-1 (Table S3), and slightly increased the shoot elongation rate in Tianlong 1, but reduced the rate of shoot elongation and transformation efficiency in Jack Purple (Table 2). Adding 200 mg/L Vc in SIM and SEM only slightly increased the rate of shoot elongation when GA<sub>3</sub> was 0.5 mg/L but not at 1 mg/L GA<sub>3</sub> for Jack Purple and Tianlong 1 (Table 3). Therefore, the effect of AgNO<sub>3</sub> and Vc on the shoot elongation rate and transformation efficiency would depend on soybean genotype and GA<sub>3</sub> concentration.

TABLE 5 | The copy number of *bar* gene in T<sub>1</sub> transgenic soybean plants.

Sample <sup>a</sup>	C <sub>t,X</sub> <sup>b</sup>	C <sub>t,R</sub> <sup>c</sup>	Ratio of <i>bar</i> /lectin (X <sub>0</sub> /R <sub>0</sub> ) <sup>d</sup>	Copy number of <i>bar</i> gene
1	26.94 ± 0.12	20.52 ± 0.00	3.10	3
2	26.24 ± 0.39	20.78 ± 0.56	1.55	2
3	26.74 ± 0.11	20.17 ± 0.21	1.42	1
4	25.51 ± 0.24	21.58 ± 0.09	1.56	2
5	24.39 ± 0.17	21.52 ± 0.23	3.42	3
6	26.54 ± 0.08	21.85 ± 0.15	0.88	1

<sup>a</sup>1–3, positive T<sub>1</sub> transgenic soybean plants in Jack Purple background. 4–6, positive T<sub>1</sub> transgenic soybean plants in Tianlong 1 background.

<sup>b</sup>C<sub>t,X</sub> represents the Ct value of the exogenous target gene (*bar*).

<sup>c</sup>C<sub>t,R</sub> represents the Ct value of the internal reference gene (lectin).

<sup>d</sup>X<sub>0</sub>/R<sub>0</sub> is the ratio of initial amount of *bar*/lectin.  $X_0/R_0 = 10^{[(C_{t,X} - IX)/SX] - [(C_{t,R} - IR)/SR]}$ , where IX and SX is the intercept and slope of the standard curve of the target *bar* gene, respectively, and IR and SR is the intercept and slope of the standard curve of the reference lectin gene, respectively.

In this study, we used four different methods to detect the positive transgenic plants, including GUS histochemical staining, PCR amplification of exogenous gene, herbicide (glufosinate) painting, and LibertyLink<sup>®</sup> strip detection. GUS staining during rooting stage is recommended to identify positive transgenic plants, which can reduce labor intensity by eliminating negative plants as soon as possible, and herbicide painting would be an easy and reliable method to confirm the results. GUS staining after transplanting might miss the detection of some positive plants, which is likely due to the fact that older leaves are difficult to get stained.

Southern blot has been a traditional method to detect the copy number of exogenous gene in transgenic plants, which gives us highly precise and intuitive results, but complex operations and large amounts of plant material are required. Absolute quantitative PCR technology provides a new approach to detect the copy number of integrated exogenous gene in transgenic plants (Ingham et al., 2001; Weng et al., 2004), which has many advantages such as lower cost, simple operation, high sensitivity and stability, and has been successfully applied to cotton (Yang, 2012), wheat (Gadaleta et al., 2011), rice (Wei et al., 2011), maize (Yuan et al., 2010), tomato (Wang et al., 2011), and soybean (Qiu et al., 2012). In this study, we used absolute quantitative PCR to detect the copy number of exogenous gene (*bar*) in the T<sub>1</sub> generation of transgenic soybean plants, and the results showed that the copy number of *bar* gene ranged from one to three. The copy number of exogenous gene in transgenic plants affects the expression level and genetic stability of the exogenous gene. The integration of multiple copies of exogenous DNA into one or more chromosomes might result in low gene expression level, low genetic stability, or even gene silencing (Iyer and Kumpatla, 2000; James et al., 2002). The ideal copy number of target gene in transgenic plants is generally one or two (Tang et al., 2007). In this study, four out of six (67%) transgenic plants contained low copy numbers (one or two), which suggests that the *Agrobacterium*-mediated transformation is a preferred method for soybean transformation.

## CONCLUSION

In this study, the *Agrobacterium*-mediated transformation efficiency in soybean was improved by increasing both *Agrobacterium* infection efficiency and explant regeneration efficiency. The *Agrobacterium* infection efficiency was more than 96% when collecting the *Agrobacterium* at a concentration of OD<sub>650</sub> = 0.6, then re-suspended in liquid CCM containing 154.2 mg/L DTT to infect the half-seed cotyledonary explants (from mature seeds imbibed for 1 day), and co-cultured them for 5 days, using the soybean varieties of Jack Purple or Tianlong 1. The shoot elongation rate of Jack Purple and Tianlong 1 increased to 33.54 and 26.08% when 1.0 mg/L GA<sub>3</sub> and 0.1 mg/L IAA were added to SEM, which is almost twice of the previous shoot elongation rate (16.11 and 14.75% for Jack Purple and Tianlong 1, respectively) with 0.5 mg/L GA<sub>3</sub> and 0.1 mg/L IAA in SEM. Ultimately, the transformation efficiency was improved from 5.00 to 7.32% and 4.28 to 10.01% for Jack Purple and Tianlong 1, respectively. This study provides an optimized *Agrobacterium*-mediated transformation protocol for soybean varieties of Jack Purple and Tianlong 1, and would be a useful reference for improving transformation efficiencies in other plant species.

## AUTHOR CONTRIBUTIONS

SL, YC, and YLi conceived and designed the experiments. SL, YC, YLiu, TW, QS, and NC performed the experiments. SL, YC, and YLi analyzed the data. SL, YC, and YLi generated the pictures. SL and YLi wrote and revised the manuscript. JG and YLi contributed reagents/materials and interpretation of the results. All authors read, revised and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2017.00246/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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