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Full Length Article

Agrobacterium-Mediated Genetic Transformation System of *Amorpha fruticosa* using Callus from the Cotyledonary Node

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Abstract

We used *Agrobacterium*-mediated infection of callus induced from the cotyledonary nodes of *Amorpha fruticosa* L. to study the *β*-glucuronidase gene (GUS)-integrated genetic transformation system. Transformed calluses were selected under 40 mg·L^{-1} kanamycin, differentiated into resistant adventitious buds, and developed into transformants. A single copy of gus was integrated in the genome of most T0 generation plants. Gus chemical staining analysis showed blue color in resistant calluses, adventitious buds, and the roots and leaves of transformed plants. This indicated gus overexpression driven by the 35S promotor and resultant *β*-glucuronidase activity. The genetic transformation system in this study could be used to study other functional genes of *A. fruticosa* and facilitate transgenic breeding for strain improvement. © 2022 Friends Science Publishers

Keywords: *Amorpha fruticosa* L.; *Agrobacterium*; Cotyledonary node; Callus; Genetic transformation

Introduction

Amorpha fruticosa L. (Fabaceae) is native to North America. It was introduced into China for use as a windbreak, for soil erosion control, and as an ornamental plant (Wang *et al*. 2002). *A. fruticosa* is not only an important landscape ecological tree species, but also a commodity tree species with high economic value. Its extracts contains biologically active substances of medicinal value. *A. fruticosa* has been used as an herbal medicine to treat fever, burns, purulent edema, and eczema in China. Induction of cell division has been used to study the activity of flavonoids extracted from *A. fruticosa* leaves. These flavonoids have potential value in new drug development (Hovanet *et al*. 2015). Fifteen medicinal ingredients including glucopyranoside, vitexin, and chrysoeriol have been isolated from *A. fruticosa* leaves (Cui *et al*. 2017). Development of new *A. fruticosa* varieties is normally accomplished using conventional breeding but transgenic technology provides an alternative means for genetic improvement of *A. fruticosa*. Plant genetic transformation is an important aspect of genetic engineering technology. It is defined as a series of events starting from the selection of required genes, delivery, integration into plant cells, expression and finally the production of the whole plant (Choudhury and Rajam 2021). Guan and Luo (2009) developed a regeneration system for callus induced in the stem segements of *A. fruticosa*.

The *Agrobacterium*-mediated genetic transformation system has often used the GUS for the genetic analysis. Jefferson *et al*. (1987) cloned GUS from Escherichia coli strain K-12. GUS is commonly used as a reporter gene in plant genetic transformation. *β*-glucuronidase is characterized by high stability, wide pH range and easily detected activity. It catalyzes the X-Gluc hydrolysis reaction and produces dark blue compounds (which are presented as blue spots) in plants. This facilitates evaluation of transformation effects or transformation efficiency (Shimomura *et al*. 1962). To develop a basic method for the study of functional genes and new line development of *A. fruticosa*, we used *Agrobacterium* to mediate GUS transformation. In this research we infected callus induced from cotyledonary nodes of *A. fruticosa* to establish an effective genetic transformation system.

Materials and Methods

Experimental material

Plant materials (*A. fruticosa* seeds) were gifts of Wu

Songquan, School of Agriculture of Yanbian University (Jilin Province, China). The bacterial strain used, *Agrobacterium* tumefaciens EHA105, carried the pBI121-GUS plasmid were available stored in our laboratory. Cetyltrimethyl ammonium bromide (CTAB), deoxynucleoside triphosphate (dNTP), and Taq polymerase, were purchased from TaKaRa Biotechnology (Dalian) (Liaoning Province, China). Kana and X-Gluc were purchased from Promega (Beijing, China). Carbenicillin disodium (Carb) was purchased from Sangon Biotech (Shanghai, China). The 6-benzylaminopurine (6-BA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthylacetic acid (NAA), Kinetin (KT), and 1-(2-chloro-4-pyridyl)-3-phenylurea (CPPU) were purchased from Nachuan (Harbin, China). Culture media composition were as follows:

(1) The medium for callus induction of the cotyledonary node of *A. fruticosa* contained MS Medium (MS) + 6-BA 3.0 $mg{\cdot}L^{-1}$ + NAA 1.0 $mg{\cdot}L^{-1}$ + 2,4-D 0.5 $mg{\cdot}L^{-1}$;
(2) The co-culture medium was the callus induction medium plus acetosyringone (AS), containing MS + 6-BA 3.0 $mg{\cdot}L^{-1}$ + NAA 1.0 $mg{\cdot}L^{-1}$ + 2,4-D 0.5 $mg{\cdot}L^{-1}$ + 20 $\mu mol{\cdot}L^{-1}$ AS;
(3) The callus induction and screening medium contained MS + 6-BA 3.0 $mg{\cdot}L^{-1}$ + NAA 1.0 $mg{\cdot}L^{-1}$ + 2,4-D 0.5 $mg{\cdot}L^{-1}$+ 40 $mg{\cdot}L^{-1}$ Kana + 500 $mg{\cdot}L^{-1}$ Carb;
(4) Screening medium for differentiation of adventitious buds from callus contained MS + 2 $mg{\cdot}L^{-1}$CPPU + 2 $mg{\cdot}L^{-1}$ KT + 40 $mg{\cdot}L^{-1}$ Kana+ 500 $mg{\cdot}L^{-1}$ Carb;
(5) The rooting medium contained 1/2 MS + 0.1 $mg{\cdot}L^{-1}$ NAA + 40 $mg{\cdot}L^{-1}$ Kana.

Experimental methods

Callus induction in *A. fruticose*: Full-size mature seeds were sterilized with 70% (v/v) alcohol and 5% sodium hypochlorite and inoculated into 1/2 MS medium for germination. When the two cotyledons unfolded, the hypocotyl was cut. The separated cotyledons were placed face up on the callus induction medium, followed by incubation at 23–25°C in a tissue culture chamber with an illumination intensity of 54 $\mu mol\ m^{-2}s^{-1}$ and a 14:10 (L:D) photoperiod.

***Agrobacterium*-mediated transformation:** A single colony of *Agrobacteria* containing the plasmid of interest (pBI121-GUS) was picked and cultured in 100 mL yeast extract peptone (YEP) medium containing 50 $mg{\cdot}L^{-1}$ Kana + 100 $mg{\cdot}L^{-1}$ Rifampicin at 28°C and 140 rpm for 48 h. Once the bacteria were grown to approximately $OD_{\lambda}600$ = 0.5 (measured by ultraviolet (UV)-spectrophotometry), AS at a final concentration of 20 $\mu mol{\cdot}L^{-1}$ and 1:10,000 (v/v) Triton X-100 were added to the bacterial culture, which was then used to immerse the calli of the cotyledonary nodes. After 10–15 min of infection, the calli were placed on sterile filter paper to remove excess bacterial liquid (Guan *et al*. 2019) and incubated with the co-culture medium at 25 ± 2°C in darkness for 3 d. Subsequently, the calli were inoculated into the callus induction and screening medium and grown in the tissue culture chamber (under the same conditions as previously described). Resistant calli were inoculated into the adventitious bud differentiation screening medium to screen the regenerated and transformed buds. The differentiated resistant buds were rooted and cultured in plant rooting medium to become resistant regenerated plants. After ventilating for acclimatization, the seedlings in the culture pots were moved into the plant culture room, the culture conditions were set as the culture temperature of 25 ± 1°C, photosynthetically active radiation of 57 $\mu mol/m^{-2}s^{-1}$ (cool-white fluorescent lamps as light resource), and an artificial 10/14 h light/dark cycle.

DNA extraction and PCR: Genomic DNA extraction was performed using the CTAB method (Sambrook and Russell 2006). The genomic DNA of the Kana-resistant plants was extracted and the wild-type *A. fruitcosa* genomic DNA was used as a control. DNA template at 1/100 dilution was used to perform PCR to amplify 5,791–7,747 bp PCR products using GUS-Forward and GUS-Reverse (GUS-F, R) primers. These were designed according to the pBI121-GUS sequence to detect the GUS integration. The PCR products were separated by 0.8% agarose gel electrophoresis.

Southern blot analysis of insert copy number of the transgenic lines: The genomic DNA (10 μg) of the leaves of GUS transgenic *A. fruitcosa* lines #1, #5, #6, #18, and #20 at the T0 generation extracted by CTAB method was incubated with HindIII/BamHI restriction enyzmes overnight at 37°C, followed by separating the cleavage products by 1.2% agarose gel electrophoresis (45 V) for approximately 9 h. A gel imager was used to detect the enzymatic digestion of the DNA. After denaturation, membrane transferring, DIG-labeled GUS (573 bp) probe hybridization (DIG-labeling kit purchased from Roche), membrane washing, and developing in CDP-Star™ reagent (Roche), the membranes were placed on the Image Quant LAS 4000 imaging analyzer (GE Healthcare Life Sciences in Germany) for signal detection (Agrawal *et al*. 2000).

Northern blot analysis of the integrated GUS expression in *A. fruitcosa* at T0 generation: Total RNA of the GUS transgenic *A. fruitcosa* lines #1, #5, #6, #18, #20 at the T0 generation was prepared by the Biozol one-step method. Five micrograms total RNA was then denatured at 65°C for 10 min and subsequently separated by 1.5% formaldehyde-agarose gel electrophoresis, followed by transferring the RNA onto a Hybond-N^+ nitrocellulose membrane. The RNA was cross-linked by UV irradiation on the membrane, followed by DIG-GUS DNA probe hybridization at 50°C for 12 h and developing in CDP-Star™ reagent before signal detection by LAS 4000 imaging analyzer (Mamiatis *et al*. 1985).

Histochemical staining analysis of β-glucuronidase activity in the genetic transformants under the 35S promoter: GUS histochemical staining was performed as described by Jefferson (1987) and Sieburth and Meyerowitz (1997). The materials were first soaked in a buffer containing 100 mM sodium phosphate buffer pH 7.0, 0.5 $mmol{\cdot}L^{-1}$

potassium ferrocyanide, and 0.5 mmol·L^{-1} potassium ferricyanide. The materials included the calli induced by transfection of pBI121-GUS plasmids, the resistant buds on the differentiation screening medium, and the roots and leaves of resistant regenerated plants. Wild-type (WT) corresponding tissues were used as control. After rinsing, the GUS staining solution (50 mmol·L^{-1} sodium phosphate buffer pH 7.0 containing 0.5 mmol·L^{-1} $K_3[Fe(CN)_6]$, 0.5 mmol·L^{-1} $K_4[Fe(CN)_6]$, 10 mmol·$L^{-1}Na_2EDTA$, 0.1% (v/v) Triton X-100, 20% methanol, and 0.5mg·mL^{-1} X-Gluc) was incubated at 37°C overnight and then the staining was observed.

Results

GUS transgenic *A. fruitcosa*: line establishment and PCR results

After soaking and infecting the callus of *A. fruticosa* cotyledons with pBI121-GUS, the resistant calli were selected by induction, and the Kana-resistant buds were selected by adventitious bud differentiation. The rooting culture was selected to obtain transformed seedlings. The acclimated seedlings were cultivated in pots and grown into GUS transgenic lines with GUS overexpression (Fig. 1).

To analyze the 35S-GUS integration of Kana-resistant regenerated plant lines (T_0), the CTAB method was used to randomly extract the leaf genomic DNA of 13 lines of transgenic resistant plants, followed by using GUS detection primers (GUS-*F, R*) to perform PCR and separating the PCR products by 0.8% agarose gel electrophoresis. In (Fig. 2), lanes 1, 3, 4 and 6–13 (positive controls) of the agarose gel show the integrated GUS DNA fragment of approximately 500 bp size; while no target DNA was detected in lanes WT, 2, and 5. WT was a negative control, and the DNA sample of the lane1 was the positive control. The #1, #3, #4, and #6–13 lines of *A. fruticosa* had integrated GUS; while the #2 and #5 lines of *A. fruticosa* had no integrated GUS.

Gene insertion copy number and overexpression in the GUS transgenic *A. fruitcosa* lines

We subjected 10 µg of genomic DNA extracted from the leaves of GUS transgenic seedlings by CTAB methods to BamHI/HindIII restriction enzyme digestion, agarose gel electrophoresis (Fig. 3), membrane transfer, and DIG-labeling Southern blot analysis. The CDP Star signal of the Southern blot was detected by LAS 4000. Figure 3B shows that the transgenic lines T0#1, #3 and #5 had a single-band signal, suggesting that a single copy of GUS was inserted into the genomic DNA of *A. fruticosa* in these lines. However, the transgenic lines T0#4 in the Southern blot had a double-band signal, suggesting that two GUS copies were inserted into the genomic DNA of *A. fruticosa* in the T0#4 line. Most of the GUS transgenic *A. fruticosa* lines had a single GUS gene insertion into the genomic DNA. This confirmed that GUS, mediated by *Agrobacterium* transformation, was successfully integrated into the *A. fruticosa* chromosome. We used DIG-GUS labeled Northern blot to detect the GUS transgenic *A. fruticosa* lines in the T0 generation (T0#1, #3, #5). The CDP-Star™ signal showed that WT had no expression compared with the GUS overexpressing lines. The GUS transgenic lines (T0#1, #3, #5) had a single band of hybridization signal, indicating that the GUS transgenic *A. fruticosa* lines at the T0 generation expressed mRNA of the exogenous GUS gene (Fig. 4). The protein synthesized by the translation of GUS expression was *β*-galactosidas and GUS staining was an effective method to detect *β*-galactosidase activity.

Activity of *β*-galactosidase expressed from the 35S promoter in resistant callus, adventitious buds, and transgenic lines

The Kana-resistant and non-transgenic calli containing pBI121-GUS plasmid after the *Agrobacterium*-mediated infection were subjected to GUS staining at 37°C overnight. The surface of most transgenic calli was blue and only a few calli were not stained. Most of the transgenic calli were stained in blue and the control calli without *Agrobacterium*-mediated infection were yellowish-white (Fig. 5). The transgenic calli stained blue confirmed the transient expression and successful plant transformation. The GUS staining analysis of transgenic resistant adventitious buds and differentiated WT-adventitious buds showed that the transgenic resistant adventitious buds were stained blue. The WT-adventitious buds were not stained, indicating that the resistant adventitious buds had GUS transgene expression and *β*-galactosidase activity. In addition, GUS staining of roots and leaves of regenerated plants lines grown by rooting culture of resistant adventitious buds showed blue color (Fig. 5C and 5D), indicating that 35S-GUS-integrated transgenic plants overexpress GUS. The 35S promoters triggered an increase in *β*-galactosidase activity. These results demonstrate the feasibility of using *Agrobacterium*-mediated infection of callus induced from cotyledons of *A. fruticosa* to accomplish genetic transformation.

Discussion

Over the years, the application of genetic transformation in *A. fruticosa* has developed steadily, which shows that the plant can successfully carry out genetic engineering and combine the characteristics of interest. Genetic engineering technology has enabled efficient genetic transformation systems for plants. Transformation can be used to analyze gene function in combination with gene-knockout technology (Yang and Zhou 2005). Although there are many developments in transgenic *A fruticosa* technology in different countries, it lags behind many other important crops. *Arabidopsis* (Clough and Bent 1998) and rice (Toki *et al*. 2006) are model plants for molecular biology research because their genetic transformants are stable. Due to its strong adaptability, *A. fruticosa* can grow at minus 40°C and

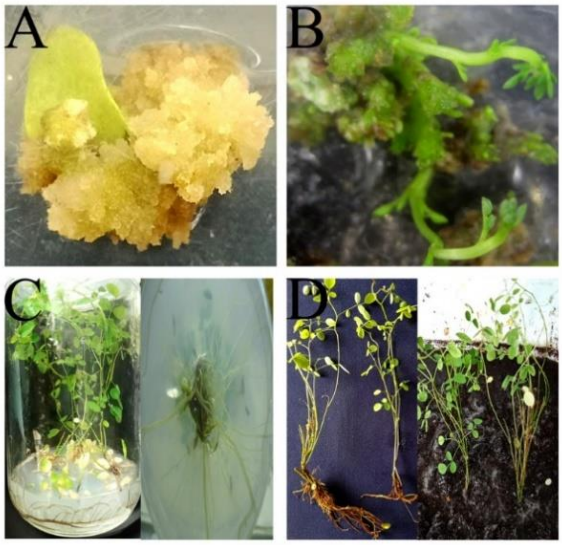

Fig. 1: Callus infection of the cotyledonary node of *A. fruticosa* for the transformation of regenerated plants. (A) Cotyledonary node-induced calli; (B) Calli differentiated into resistant adventitious buds; (C) Regenerated lines of resistant adventitious buds from rooting culture; (D) Transgenic seedlings of the regenerated lines from soil culture

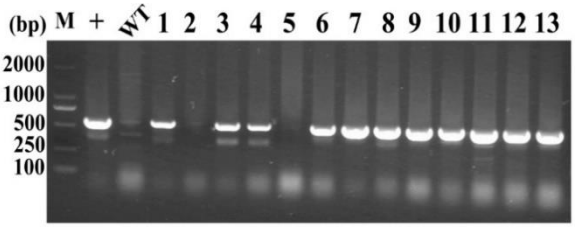

Fig. 2: PCR detection of GUS transgenic lines. WT represents a negative control with a DNA template from non-transgenic plant. "+" represents the positive control with plasmid DNA template; lanes 1–13 represent DNA templates from transgenic plant lines

where the annual precipitation is only about 200mm. Its ability to resist flooding, salt and alkali, barren, wind and sand, insects, tobacco and pollution is very rare in plant populations (Sun *et al*. 2021). It is desirable to establish a genetic transformation system for *A. fruticosa*. Stable transformation can transfer integrated genes in successive generations and meet the requirements of functional genomics and transgenic breeding (Choudhury and Rajam 2021). Establish an efficient and high-throughput transformation system for *A. fruticosa* plants, and finally introduce the required characters into the plants, so as to improve their yield. A genetic transformation receptor system with efficient and stable regenerative capacity, sensitivity to selective antibiotics, and sensitivity to *Agrobacterium* infection is required for completing gene transfer.

Selection pressure of Kana (40 $mg \cdot L^{-1}$) was used to differentiate the calli of the cotyledons of *A. fruticosa*

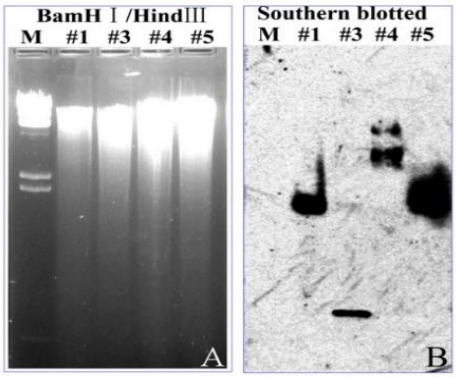

Fig. 3: DIG-GUS labeled Northern blot was used to detect the GUS transgenic *A. fruticosa* line at the T0 generation (T0#1, #3, #4, and #5). The CDP-Star™ signal showed that WT had no expression and GUS transgenic lines (T0#1, #3, #4, and #5) had hybridization signals, indicating that the transgenic *A. fruticosa* lines at the

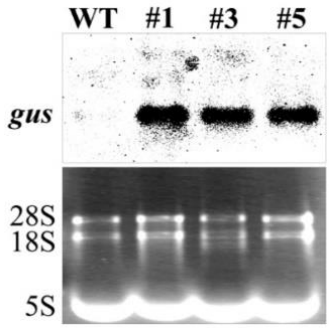

Fig. 4: Gene expression signal detected by Northern blot. The #1, #3, and #5 are the numbers of transgenic plants

infected by *Agrobacteria* containing GUS into resistant adventitious buds. Molecular testing revealed the single- and double-copy insertions in the regenerated plants (Fig. 3). In addition, GUS at the mRNA level was overexpressed by the 35S promoter. However, the type of integration was unrelated to the activity of the translated protein (Papadopoulou *et al*. 2005). GUS encodes *β*-glucuronidase, which hydrolyzes X-gluc and produces a blue color (Lambé *et al*. 1995). Detection of *β*-glucuronidase activity in the transgenic lines reflects the expression of GUS-encoded protein (Yancheva *et al*. 1994). Recently, it has been reported that in addition to transforming *Agrobacterium* strains, there are many modified bacterial species of plants. Such as *Sinorhizobium meliloti*, *Mesorhizobium loti*, *ensifer adhaerens*, in which *S. meliloti* can infect monocotyledons and dicotyledons (Rathore and Mullins 2018). In this research, 35S promoter driven Gus overexpression and production *β*-glucuronidase activity provides a new direction for transgenic breeding of improved strains.

In conclusion, this study achieved the goal of successful gene editing and stable transformation of *A. fruticosa*. The scheme of *Agrobacterium* mediated genetic

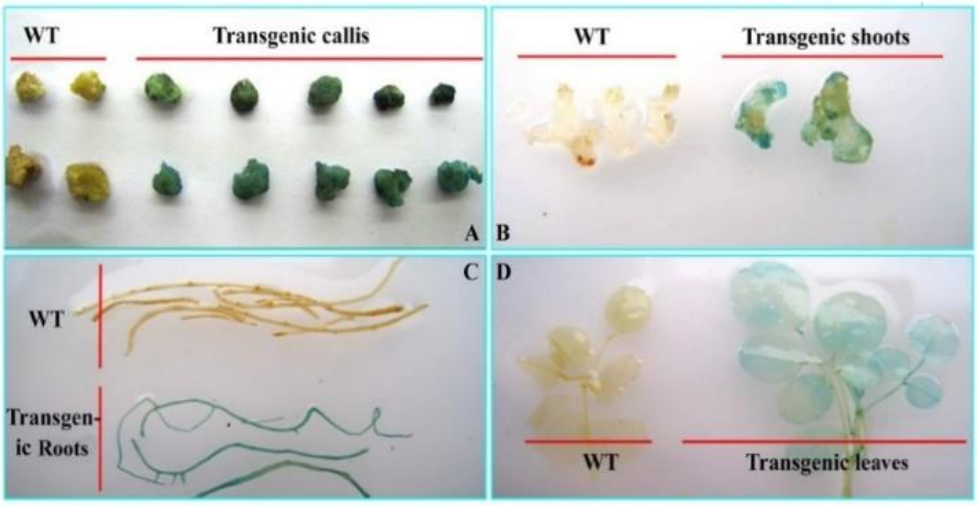

Fig. 5: GUS histochemical staining of the process of genetic transformation of *A. fruticosa* by *Agrobacterial* infection. (A) GUS transient expression of the transgenic calli; (B) GUS expression in resistant adventitious buds; (C and D) GUS expression in the roots and leaves of the transgenic lines

transformation of *A. fruticosa* was optimized. It is expected that a major breakthrough in the genetic improvement of *A. fruticosa* is no longer far away. Therefore, this method can play an important role in the functional genomics of *A. fruticosa* gene and release the real potential of gene editing in the production of improved *A. fruticosa* varieties.

Conclusion

We used GUS histochemical staining to detect *β*-glucuronidase activity in callus, adventitious buds, and transgenic lines during *A. fruticose* transformation and regeneration. The staining verified successful establishment of a genetic transformation system with efficient and stable regenerative capacity, sensitivity to selective antibiotics, and sensitivity to *Agrobacterium* infection of *A. fruticosa.*

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Author Contributions

Qingjie Guan conceived and designed the study. Yiteng Zhang and Jiali Liu performed the experiments and drafted the manuscript. Ailing Zhong and Ziang Liu contributed to the sample measurement and data analysis. XiuFeng Li, Kai Wang, Zhenyu Wang and Minghui Li draft revision. All authors read and approved the final manuscript.

Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author

Ethics Approval

Not applicable in this paper

References

Agrawal GK, NS Jwa, R Rakwal (2000). A novel rice (*Oryza sativa L.*) acidic PR1 gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors. *Biochem Biophys Res Commun* 274:157–165

Choudhury A, MV Rajam (2021). Genetic transformation of legumes: an update. *Plant Cell Rep* 40:1813–1830

Cui X, J Guo, CS Lai, MH Pan, Z Ma, S Guo, Q Liu, L Zhang, CT Ho, N Bai (2017). Analysis of bioactive constituents from the leaves of *Amorpha fruticosa* L. *J Food Drug Anal* 25:992–999

Clough SJ, AF Bent (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743

Guan QJ, QX Luo (2009). Induction of callus and plant regeneration for stem segments of *Amorpha fruticosa*. *Liaoning For Sci Technol* 6:8–11

Guan QJ, ML He, HY Ma, X Liao, ZJ Wang, S Liu (2019). Correction to: Construction of genetic transformation system of salix mongolica: *In vitro* leaf-based callus induction, adventitious buds differentiation, and plant regeneration. *Plant Cell Tiss Org Cult* 136:205–205

Hovanet MV, IC Marinas, M Dinu, E Oprea, V Lazar (2015). The phytotoxicity and antimicrobial activity of *amorpha fruticosa l*. leaves extract. *Romanian Biotechnol Lett* 20:10670–10678

Jefferson RA, TA Kavanagh, MW Bevan (1987). GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO J* 6:3901–3907

Lambé P, M Dinant, F René, RF Matagne (1995). Differential long-term expression and methylation of the hygromycin phosphotransferase (hph) and β-glucuronidase (gus) genes in transgenic pearl millet (*pennisetum glaucum*) callus. *Plant Sci* 108:51–62

Mamiatis T, EF Fritsch, J Sambrook, J Engel (1985). *Molecular Cloning-a Laboratory Manual*. Cold Spring Harbor Laboratory, New York, USA

Papadopoulou KK, N Kavroulakis, M Tourn, I Aggelou (2005). Use of β-glucuronidase activity to quantify the growth of *fusarium oxysporum* f. sp. *radicis - lycopersici* during infection of tomato. *J Phytopathol* 153:325–332

Rathore DS, E Mullins (2018) Alternative non-*Agrobacterium* based methods for plant transformation. *Annu Plant Rev Online* 1:891–908

Sambrook J, DW Russell (2006). Detection of protein-protein interactions using the GST fusion protein pulldown technique. *CSH Protoc*. 2006:pdb.prot3757

Shimomura O, FH Johnson, Y Saiga (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J Cell Compar Physiol* 59:223–239

Sieburth LE, EM Meyerowitz (1997). Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *Plant Cell* 9:355–365

Sun X, S Hu, X Wang, H Liu, YW Zhou, Q Guan (2021). *De novo* assembly of *Amorpha fruticose* L. transcriptome in response to drought stress provides insight into the tolerance mechanisms. *Peer J* 9:e11044

Toki S, N Hara, K Ono, H Onodera, A Tagiri, S Oka, H Tanaka (2006). Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. *Plant J* 47:969–976

Wang ET, MA Rogel, XH Sui, WX Chen, E Martínez-Romero, P van Berkum (2002). *Mesorhizobium amorphae*, a rhizobial species that nodulates *Amorpha fruticosa*, is native to American soils. *Arch Microbiol* 178:301–305

Yancheva SD, M Vlahova, P Vlahova, A Gercheva, Atanassov (1994). *Agrobacterium* mediated transient expression of *β*-glucuronidase gus gene in plum (*Prunus domestica* L.). *Biotechnol Biotechnol Equip* 8:12–13

Yang YJ, P Zhou (2005). Advances in the study on marker genes in transgenic plants. *Yi Chuan* 27:499–504