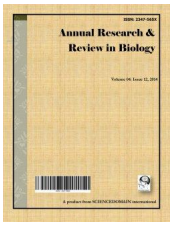

Annual Research & Review in Biology

30(1): 1-7, 2018; Article no.ARRB.46049
ISSN: 2347-565X, NLM ID: 101632869

Agrobacterium-Mediated Transient Assay of the *Gus* Gene Expression in Sugar Beet

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Authors' contributions

This work was carried out in collaboration between all authors. Author KM performed the experiment and the statistical analysis and wrote the first draft of the manuscript. Authors SEM and BH wrote the protocol, designed the study and revised the final manuscript. Author PN provided the seeds and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/46049
Editor(s):
(1) Dr. Jin-Zhi Zhang, Key Laboratory of Horticultural Plant Biology (Ministry of Education), College of Horticulture and Forestry Science, Huazhong Agricultural University, China.
(2) Dr. George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Taihe Xiang, Hangzhou Normal University, China.
(2) Jin Seop Bak, UCC, Cambridge.
Complete Peer review History: http://www.sciencedomain.org/review-history/27998

Original Research Article

Received 24 September 2018
Accepted 16 December 2018
Published 29 December 2018

ABSTRACT

Modification of transformation systems with a set of markers is almost used to confirm whether the transgene has been successfully transmitted to the host cells. Transient expression technique is a fast and simple way to analyze promoter expression. This method is not affected by the position of the transgene in the target genome. In the present study, the *gus* reporter gene directed by the *CaMV 35S* promoter and the *nptII* selectable gene were used for optimization of transformation event in sugar beet. The results demonstrated the activity of β-glucuronidase in the *Agrobacterium* cells showing suppressed expression of the prokaryotic reporter gene. The function of the *pCAMBIA2301* vector was assessed through inoculation of shoot apex with *Agrobacterium*. The results demonstrated that cells adjacent to the main vein of leave reared from tissue cultured apical meristems were suitable for transformation and regeneration. The highest shoot regeneration was

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achieved for tissue-cultured leaf explants grown in the presence of BA, IBA and TDZ media. In this study, an improved protocol for regeneration and genetic engineering of a sugar beet genotype was described using the tested vector. Analysis of GUS Histochemical and polymerase chain reaction (PCR) of the T0 generation plants demonstrated that the tested vector enables the expression of the *gus* gene in the transgenic plants that was an evidence of transient expression.

Keywords: Transformation; CaMV 35S promoter; Agrobacterium; gus reporter gene.

1. INTRODUCTION

Transient gene transformation techniques are used to set up transformation systems. In transient gene expression techniques, a transgene is expressed for a short time after transfer into the host plant cells. In transient assays, insertion of the transgene in the genome is not necessary and the transgene will not inherit. The transient expression method has caught attentions in gene transformation studies [1-2-3]. Its simplicity and time-effective are two major advantages of transient expression techniques whereas lack of constant transfer of the system to the next generation could be a demerit [4]. On the other side, considering biosafety issues such disadvantages can be environmentally an advantage [5]. Transient expression of a gene in the target cell is feasible via different methods including use *Agrobacterium*- mediated methods [6-3-2]. **I**n transient expression method, a vector harboring reporter and selective genes with low background activity in plants is needed. The product has moderate stability *in vivo* with down regulation of gene [7]. In the promoter analysis studies, it is possible to compare the level of expression of promoters with a control sample given the vector harboring the sequence of CaMV35S is available as a positive control. Moreover, the use of a reporter gene containing an intron makes it possible to distinguish between eukaryotic and prokaryotic expressions and it prevents from intervention of bacterial expression in the resultant transformed samples [5].

Sugar beet (*Beta vulgaris* L.) is important in sugar industry. This species is one of important crops from which sucrose (i.e., sugar) can be economically produced [8-9]. This species is not only considered as a source of sugar but also as a green bioreactor for the storage of new metabolites in the root [10-11]. Given the high-energy value of intermediates of sugar beet processing, sugar beet products can be used as raw materials for alcohol, ethylene, citric acid, glutamic acid, dough, antibiotics, vitamins and resins [12-13-14]. Sugar beet pulp is used to produce pectin and galactone acid and indirectly in the production of vitamin C. However, cross-pollination and biannual development characteristics combined with a high level of heterozygosity make the process of production of new varieties via classical breeding techniques long. Traditional breeding has played a vital role for productivity improvement in sugar beet. More recently, molecular biotechnological approaches have been developed and integrated with the conventional approaches. This leads to enhanced efficiency of conventional sugar beet breeding through integration of molecular marker-assisted selection and development of novel sugar beet strains through genetic engineering [15]. *Agrobacterium*-mediated transformation is much simpler than other techniques of gene transformation and unlike stable transformation allows the analysis of deleterious effects of genes on growth and development [2]. Given the importance of sugar beet breeding, the main objective of the present study was to assess the efficiency of a modified protocol with high efficiency rate for gene transformation in sugar beet. The modified protocol used in the present study might assist sugar beet research community in large scale production of transgenic beet varieties.

2. MATERIALS AND METHODS

2.1 Plant Materials

A diploid sugar beet line (SBSI-11, O-type) was selected for analysis of the transient assay. This line has been used as parental line for production of hybrid varieties in the Sugar Beet Seed Institute (SBSI), Karaj, Iran.

2.2 Tissue Culture

The interested seeds were treated with concentrated H_2SO_4 while gently shaking for 30 min. Then, the seeds were rinsed with sterile distilled water and subsequently surface-sterilized in 70% (v/v) ethanol for 1 min and 5% (w/v) chlorax solution contained a drop of Tween

20 for 15 min. After each step, the seeds were rinsed carefully with sterile distilled water.

The treated seeds were sown in water–agar medium (7 g L^{-1}). The germinated seeds were transferred into sterile Petri dishes contained 35 ml MSB medium [16] and supplemented with B5 vitamins [17], 30 gL^{-1} sucrose and 7g L^{-1} plant agar. After two weeks, shoot-apex of seedlings were excised and were transferred into the shoot induction medium in which MSB medium were supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} plant agar, and 0.25 mg L^{-1} N6-benzyl adenine (BA) and 0.1 mgL^{-1} Indole-3-butyric acid (IBA) were used as plant growth regulators. After 3 weeks, the shoots were transferred into the MSB proliferation medium enriched with 0.25 mgl^{-1} N6-benzyl adenine (BA), 0.1 mg L^{-1} Indole-3-butyric acid (IBA), 0.1 mg L^{-1} thidiazuron (TDZ) as growth regulators and supplemented with 30 gL^{-1} sucrose and 7g L^{-1} agar. After 4 weeks, developed leaves were removed from the shoots and transferred to proliferation medium until adventitious shoots appeared on the mid vein. Then, the adventitious shoots were removed from the leaves and the leaves were used as explants in *Agrobacterium*-mediated gene transformation.

2.3 Bacteria, Strain and Plasmid

Agrobacterium tumefaciens strain LBA4404 [18] harboring pCAMBIA2301 was used for gene transformation. The T-DNA region of the pCAMBIA2301 vector contains neomycin phosphotransferase gene (*nptII*) as a plant selectable marker for selection on kanamycin and the β -glucuronidase (*gus*) as the reporter gene directed by cauliflower mosaic virus (CaMV 35S) promoter. A bacterial colony were grown in liquid LB medium [19] supplemented with 75 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin with shaking at 180 rpm. The cultures were incubated at 28 ℃ for two days or until the OD600 of solution met 0.5–0.7. The bacterial cultures were harvested through centrifugation at 3000 rpm under 4℃ for 15 min and then resuspended in induction medium consisted of liquid MSB medium (half- strength MS salts), supplemented with 50 g L^{-1} glucose and 50 mM acetosyringone. The pH of the medium was adjusted on 5.5.

2.4 Optimization of Transformation and Regeneration Events

The explants were immersed into the *Agrobacterium* suspension culture for 5-10 min. The explants were dried on sterile filter paper to remove excess bacteria and subsequently were co-cultured for 3 days into shoot-induction MSB medium consisting of half-strength MS salts, 50 mM acetosyringone, 30 g L^{-1} sucrose and 7 g L^{-1} agar. Then, the explants were washed for 15 min with sterile distilled water containing 500 mg L^{-1} cefotaxime while gentle shaking. The explants were placed on shoot-inducing medium containing 100 mgL^{-1} kanamycin, 250 mgL^{-1} cefotaxime, 30 g L^{-1} sucrose and 7 gL^{-1} agar, and 0.25 mg L^{-1} N6-benzyl adenine (BA), 0.1 mg L^{-1} Indole-3-butyric acid (IBA) were used as growth regulators. After 2 weeks, explants carrying regenerated shoots were transferred to a fresh shoot-inducing medium and subcultured at 2-week intervals. Then, regenerated shoots were excised and transferred into shoot growth medium comprising MSB medium supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar, 250 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin and subcultured at 2-week intervals. Then the shoots longer than 60 mm were transferred into the root-inducing medium comprising MSB medium supplemented with 20 g L^{-1} sucrose and 7 g L^{-1} agar, and 3 mg L^{-1} IBA was used as growth regulator. The rooted plantlets were transferred into growth chamber (Paradise, SPG 30000 AX) with the growth condition was considered as 20 ± 2°C as day/night temperature, 70% relative humidity, and 12/8 h light periods.

2.5 Regeneration and Screening Kanamycin-Resistant Plants

The bud induction medium was prepared through blending hormones that afforded desirable conditions for induction of large buds on the leaf area, and especially nearby the principal vein. The line used for gene transformation in the present study was highly responsive to regeneration medium. After three days of co-culture practice, transgenic buds were selected based on response to kanamycin. At different steps of selection practice, kanamycin was applied in 100 mg L^{-1} concentration and the transgenic buds were subcultured at two-week intervals. Inoculated leaves in the kanamycin medium (control sample 1) were colorless that was due to the presence of the selective marker whereas the second control sample preserved its vitality and was resumed to grow under non-kanamycine condition.

2.6 Histochemical Assay

Putative transformed buds were isolated and flooded in X-gluc solution up to 2 hours. The X-gluc solution was consisted of 1 mM substrate in

50 mM NaH2PO4, and pH of the solution was adjusted as 7.0, and the temperature of the reaction was considered as 37°C. After staining, the samples were rinsed in 70% ethanol for 5 min, and then were microscopic mounted.

2.7 PCR Analysis and Validation of Gene Transfer Events

Genomic DNA was extracted from leaf tissues as described by Štorchová [20]. To confirm the presence of the transgene in putative transgenic seedlings, PCR test using specific primers for CaMV35S promoter was performed. PCR conditions was considered as: 94°C preliminary denaturation for 5 min, 35 cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30s, extension at 72°C for 1 min, and a final extension at 72°C for 3. The PCR product was separated by electrophoresis using a 1% agarose gel, and the gel was photographed by Gel-Doc apparatus after ethidium bromide staining.

3. RESULTS AND DISCUSSION

3.1 Regeneration of Transformants

The results demonstrated that shoot apex in the sugar beet line was sensitive to kanamycin in the regeneration medium. The concentration of 100 mg L^{-1} kanamycin had a significant effect on bud induction event and adventitious bud regeneration. Therefore, this concentration of kanamycin was appropriate for the adventitious bud differentiation. This is an important issue in transformation events because kanamycin at high concentrations prevents the synthesis of chlorophyll and reduces the growth and regeneration vigor of transgenic buds [11,21]. In the present study, green explants of kanamycin-resistant samples were discriminated from non-transgenic ones (Fig. 1). The results showed that up to 50% of transgenic explants were kanamycin-resistant demonstrating the efficiency of the modified protocol used in this study. In Lindsay and Guillaume [22] study, the presence of the *gus* gene was confirmed in 30% of the kanamycin-resistant sugar beet samples only. In another transformation assay with hygromycin, the PCR- positive seedlings induced from transgenic buds varied between 15.2% and 38.7% [23]. Indirect regeneration is time-consuming event with low repeatability of regeneration. It also leads to undesirable morphological and genetic variations as a consequence of variations in media components used for regeneration [11-21]. In the present study, the shoot-apex explants were responsive to hormonal treatments and a high regeneration ratio was obtained in the medium with BA, IBA and TDZ hormones. These types of explants grew up around the main vein (Fig. 2). Young tissues almost composed of newly expanded cells from vigorously growing plants often show high level of transient expression demonstrating high physiological activity of such cells [2]. The results of this study showed that bud leaf could be an appropriate explant for transformation of sugar beet. It has also several advantages including the simplicity of explant production, high regeneration for the preparation of the target explants and reduced time for regeneration of transgenic buds. Overall, the results of our study were in line with results of other studies [21, 24, 25] with respect to high repeatability and efficiency of the modified method used for production of transformed sugar beet samples in large scale.

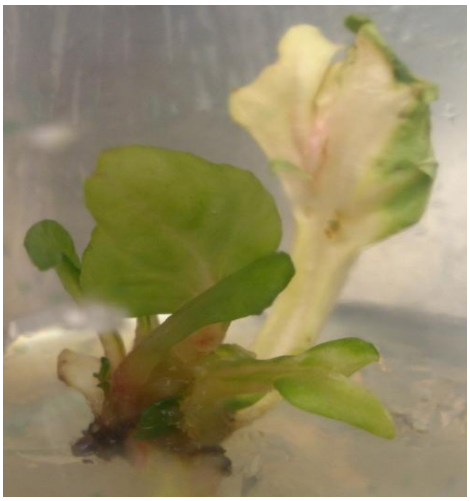

Fig. 1. Sugar beet regenerated resistant buds in 100 mg L^{-1}kanamycin (Colorless leaf associated with the presence of kanamycin) concentration.

3.2 Expression of the *Gus* Gene in Transgenic Sugar Beet

The blue colored products of GUS activity were visible after incubation for 5-24 h. No blue-colored products were detected in the tissues of the non-transformed control plants (Fig. 3). A total of 121 independent transgenic plants harboring pCAMBIA 2301 were analyzed by the histochemical staining for GUS activity. Of these, 63 samples (52.06%) showed GUS activity in bud.

3.3 Molecular Analysis of the T0 Transgenic Plants

Results of PCR confirmed the presence of the *gus* gene in kanamycin-resistant T0 transgenic plants. Transgenic explants showed the bands amplified with pCAMBIA plasmid demonstrating positive gene expression in the tested samples. The specific band was not amplified in the non-transgenic samples demonstrating the absence of the *gus* gene. More than 50% of the kanamycin-resistant explants were identified as PCR-positive samples (Fig. 4). In a study, Mohammadizadeh et al. [26] PCR was used to confirm the presence of the polygalactorinase inhibitor protein in the T0 transgenic sugar beet plants.

Fig 2. A tissue-cultured leaf explant with numerous shoots regenerated from the cells around the main vein.

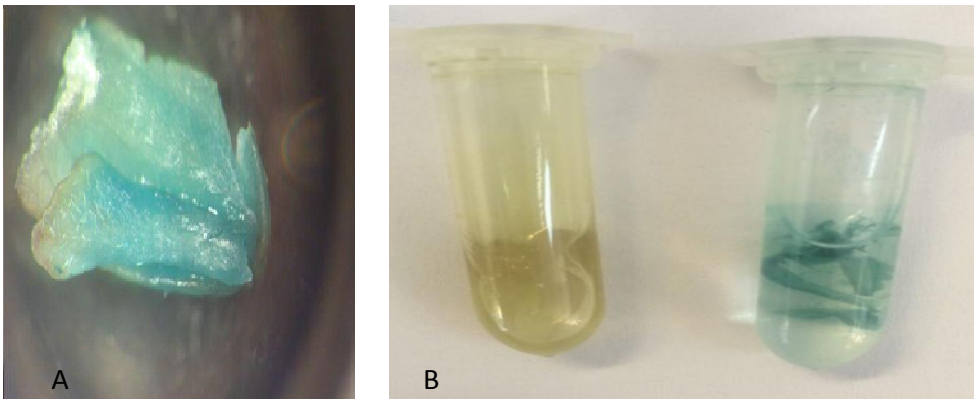

Fig. 3. Gus staining for bud regeneration

A: Expression of the GUS gene in regenerated sugar beet buds. B: comparison between non-transgenic tissues (negative control) and transgenic plant

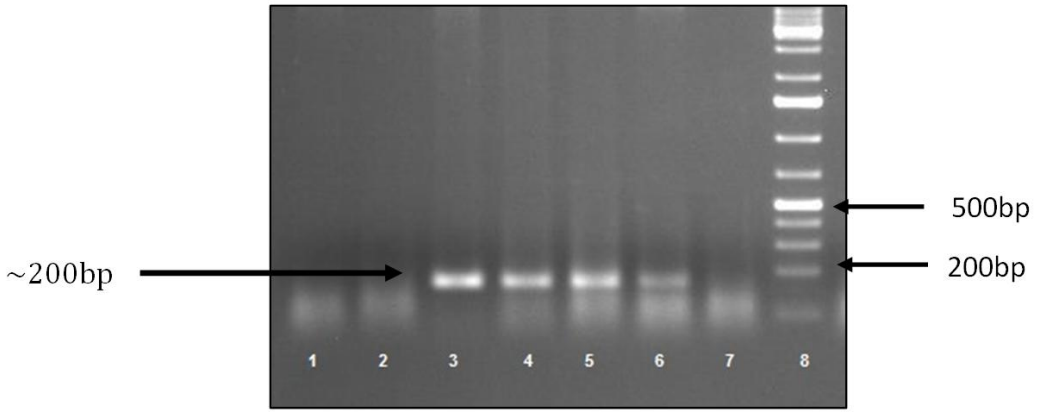

Fig 4. Polymerase chain reaction (PCR) of the transgenic sugar beet. PCR products of the transformed and non-transformed samples were analyzed on gel electrophoresis with respect to pCAMBIA2301-*gus*

Lane 1: Distilled water, Lane 2: DNA extracted from non-transgenic plant as negative control, Lane 3: positive control (plasmid containing CaMV 35S promoter), Lanes 4-7: transformed samples, Lane 8: ladder of 1 kb size.

In the present study, the efficiency of pCAMBIA2301 vector to transform sugar beet with the *gus* gene was demonstrated in a sugar beet line. The pCAMBIA2301 expression vector has been successfully used to modify plants. This vector contains the CaMV 35S promoter which is compatible with expression in leaves, fruits, tubers and roots of the dicotlydons species. The level of gene expression is the net balance of the long-term transcription and translation events. In transient assays, high level of gene product may accumulate prior to the initiation of post transcriptional gene silencing demonstrating the efficiency of this assay over stable transgene expression methods [2]. Transformation with *Agrobacterium* is still the most efficient method for the production of transgenic plants [27]. The level of transient expression often exceeds those observed in stably transgenic samples [2]. The efficiency of transformation is greatly influenced by the compatibility between plant and bacterium. Some of strains are more virulent than others [2].

4. CONCLUSION

A. tumefaciens strain LBA4404 harboring pCAMBIA2301 has been successfully used to modify plants. In the present study, this plasmid was used to transform a nematode resistant line. Transgenic plants containing the *gus* gene showed GUS activity in bud. Among transgenic plants, 52.06% showed the presence of the *gus* gene in kanamycin-resistant T0 plants. The results demonstrated that shoot apex was sensitive to kanamycin. A concentration of 100 mg L^{-1} had a significant influence on bud induction and adventitious bud regeneration. Shoot-apex explants had a good response to hormonal treatments and also a high level of regeneration was observed in the medium containing BA, IBA and TDZ hormones. Tissues producing numerous shoots, mostly located at the main vein extending from petiole to leaf blade. Overall, the results of the present study might assist sugar beet breeders with respect to the identified efficient protocol for gene transformation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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