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DEVELOPMENT OF AN EFFICIENT *AGROBACTERIUM*–MEDIATED GENE TRANSFER SYSTEM FOR THE VARIETAL IMPROVEMENT OF *SIMAROUBA GLAUCA* DC

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ABSTRACT

The genetic transformation of *Simarouba glauca* will help researchers across the globe in developing high medicinal value and high biomass varieties in a short period. Although genetic transformation methods have been trailed by many research groups for several years, the variation in transformation efficiency and difficulties in handling tissue culture plantlets made it difficult tooptimize a reliable protocol. In this study, we established an alternative protocol of genetic transformation by introducing *GUSPlus* reporter gene into the genome of an *S. glauca* through the *Agrobacterium*-mediated gene transfer method which is the first report in India. Leaf explants co-cultivated with *Agrobacterium* LBA4404 strains harbouring pCAMBIA 1305.1 vector have shown the presence of *GUS Plus* and *HptII* gene by PCR analysis as well as GUS assay. The outcome of our research provides fundamental information on efficient gene transformation in *S. glauca* which will help to enhance future research on the genetic transformation of *S. glauca*.

Keywords : *Simarouba glauca, Agrobacterium*-mediated gene transfer, PCR analysis.

Introduction

Simarouba glauca commonly known as laxmitaru or paradise tree belongs to the family Simaroubaceae and is a medium-sized evergreen tree and medicinally important plant. The oil seed of *S. glauca* is used as alternative energy to replace fossil fuels. The chemical constituents of this plantare used as antiprotozoal, anti-amoebic, and antimalarial. It is a medium-sized tree of height 7-15 meters, introduced from their native of Florida, South America, the Caribbean, and El Salvador to India. Itis spread throughout the country due to its wide adaptability to various climatic conditions and drought hardiness (Joshi Syamasundar and Joshi Shantha, 2002). The seeds of the plant contain quassinoids i.e., a group of degraded triterpene lactones, such as glaucarubol, glaucarubolone, and the two esters of glaucarubolone and ailanthinone (Kupchan *et al.,* 1975). The extract from the tree, Glaucarubinone, is generally known in India as Lakshmitaru, which is claimed as apotent anti-cancer compound (Gaudemer and Polonsky, 1965).The tree has a well-established rooting system thatcan check soil erosion and improves groundwater position and support microbial life. *S. glauca* is also known for reducing the accumulated carbon dioxide (CO_2) into oxygen (O_2) thus helping the reduction of global warming. Several plants such as *Simarouba glauca*, *Jatropha curcus*, *Saccharum officinarum, Azadirachta indica*, *Calophyllum inophyllum*, *Pongamia pinnata*, *Euphorbia tirucalli*, *Hevea brasiliensis*, *Boswellia ovalifoliolata,* and *Mahua indica* are recognized for the production of biofuel due to its high biomass content.

S. glauca has a high potential for producing biodiesel due to its high oil content (Joshi and Shantha, 2000). The seeds produced 50-65% oil that can be easily extracted using the conventional method and a well-grown tree yields 15-30 Kg nutlets per year which is equivalent to 2.5-5.0 Kg oil. This gives 1-2 tons of oil per hectare per year (Devan and Mahalakshmi, 2009). *S. glauca* is a promising feedstock for biodiesel production.There are no reports on an effective method for varietal improvement in *S. glauca* using genetic transformation.The transgenic approach has the potential to significantly improve seed productivity in *Simarouba* which in turn can help in improved biodiesel production.An alternative source for traditional breeding is genetic transformation which is a noble tool for tree improvement. Substantial efforts have been capitalized in genetic engineering in recent years for the isolation and characterization of desired genes from tree species.

Gene delivery system includes biological-based transformation and non-biological-based transformation. The biological-based method involves the use of biological material for gene transfer, such as *Agrobacterium tumefaciens*. Particle bombardment or Biolistic, as well as electroporation mediated method, are thus referred to as non-biological based transformation (Keshavareddy *et al*., 2018). Particle bombardment or biolistic method has some

disadvantages such as; they are very costly, causing DNA damage, lowest transformation efficiency, and gene silencing caused by multiple copy insertion compared to the *Agrobacterium*-mediated gene transfer system (Shunhong Dai *et al*., 2001). Many researchers have followed *Agrobacterium*-mediated gene transfer systems due to their high transformation efficiency, low cost, harmless nature, stable transformation, and eco-friendly than the other gene delivery systems.

Agrobacterium tumefaciens is a gram-negative soil pathogen that mostly infects dicotyledonous plants and causes crown gall disease due to tumorous growth at the wound site (Smith *et al*., 1907). The bacteria havea large vector that contains T-DNA (transfer DNA) which is transferred into the infected host cell along with *vir* genes stimulated by Acetosyringone exude by host wounds. Then it has integrated into the plant genome and can be replaced by any gene of interest without any adjustment of DNA sequence. As a final point, this process is followed by the central dogma. In this way, the desired gene transformation takes place in plants via *Agrobacterium*-mediated transformation (Binns *et al*., 1988; Gelvin, 2003). The leaf explants are mostly used for the transformation method (Horsch *et al*., 1985). In this experiment, the transformation process has carried out by leaf explant.

β-glucuronidase (GUS) is an enzyme encoded by *E. Coli Gus plus* gene that is mostly used as a reliable transgenic reporter system to help detect transformation events in plant tissues (Miki and McHugh, 2004). The detection of the *GUS Plus* gene can be observed easily by staining with 5-bromo-4-chloro-3-indolyl-b-D glucuronic acid (X-GLUC). The expression of the *GUS Plus* gene is a very stable, simple, cost-effective method and has no toxicity after transformation into the plant systems (Mantis *et al*., 2000). The present study describes the efficient transformation protocol for *S.glauca* using an *Agrobacterium*-mediated transformation system.

Material and Methods

Preparation of Leaf and stem explants

Young leaves as well as stems from new branches of *S. glauca* were collected from 3 to 4 years-old plants grown in the outskirts of Coimbatore, Tamil Nadu, and India. The collected leaf and stem samples were rinsed separately in running tap water for 30 min. The rinsed explants were then surface sterilized with 3.0% sodium hypochlorite (NaClO) solution for 15 min and rinsed three times with sterile distilled water. The explants were then taken under a laminar airflow chamber and treated with 70% ethanol for 5 min followed by rinsing in sterilized distilled water three times. The explants were finally treated with 0.1% mercuric chloride ($HgCl_2$) solution for 10 min and rinsed thoroughly three times with sterilized distilled water to remove any excess residues of $HgCl_2$. The sterilized leaf explants were scraped using cellophane tape to remove the waxy cuticle for easy transformation, while no extra treatment was carried out for stem explants. Both explants (leaf and stem) were excised into small pieces on a sterile Petri plate containing ascorbic acid (50mg/L) and citric acid solution (100mg/L) to remove phenolic content for co-cultivation with *Agrobacterium tumefaciens*.

Bacterial strains and plasmid

Agrobacterium tumefaciens LBA4404 strain harbouring the binary vector pCAMBIA1305.1 (Figure-1),kindly donated by Dr. C. Appunu from Division of Crop Improvement at ICAR-Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India, was used in genetic transformation experiments. The vector harbours the *Gus*Plus gene (β-glucuronidase), as well as the HptII gene and their activity, which is driven by the cauliflower mosaic virus 35S promoter (CaMV35S). *Gus* plus gene confers kanamycin resistance. A single colony of *Agrobacterium tumefaciens* LBA4404 was grown in 10 ml of YEP (An *et al*., 1989) liquid medium containing 10mg/L rifampicin and 50 mg/L kanamycin, and maintained at 28°C on a rotary shaker at 300 rpm overnight. One ml of overnight culture was transferred to 50 ml of YEP liquid medium with the same antibiotics and allowed to grow at 28°C. The well-grown bacterial culture was centrifuged at 8000rpm for 15 min and the bacterial pellet was re-suspended with 100ml of MS medium (pH 7) without agar and any hormones (Murashige and Skoog, 1962) for plant transformation.

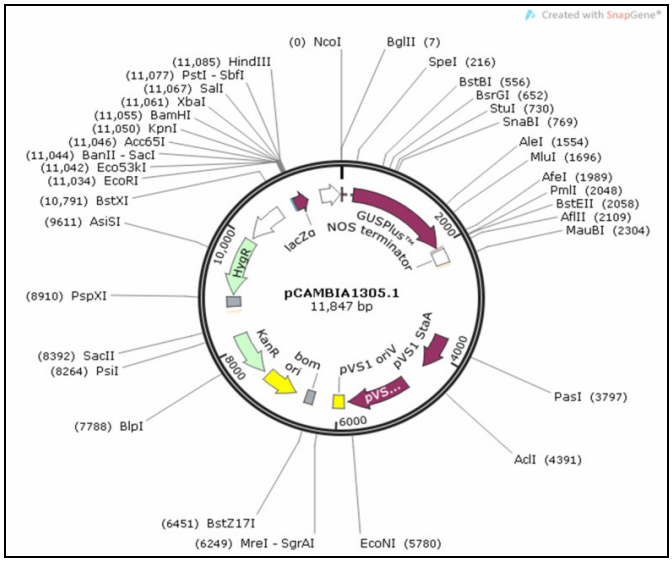


Fig. 1 : Binary vector of pCAMBIA1305.1

Transformation of *Simarouba glauca*

The prepared explants were treated with *Agrobacterium* suspension (OD_{600}=0.8, 0.7, 0.6, 0.5, 0.4, and 0.3) for 30 min for infection and dried over a sterile filter paper. The explants have sub-cultured on Murashige and Skoog (MS) medium supplemented with activated charcoal (0.1%), 3% of sucrose, 0.8% of agar, 3 mg/L of 6-benzyl amino purine (BA) and 0.01 mg/L of indole-3-butyric acid (IBA) for 3 days in the dark at 25±2°C. The co-cultivated explants were treated with 500mg/L cefotaxime solution to remove the excess growth of bacteria. After a week, the explants were transferred to MS medium supplemented with 10mg/L rifampicin, 50 mg/L kanamycin, 10mg/L hygromycin, and maintained under 12 h light/12 h dark cycle at 25 ± 2°C.

Histochemical analysis of *GUS* Activity:

GUS histochemical assay was carried out by the method of Jefferson *et al*, (1987). The selected explants were treated with 1mM X-gluc (5-bromo-4-chloro-3-indolyl-β-glucuronic acid)solution with a composition of 50 mM Na_2HPO_4, 10 mM K_4 Fe (CN), 03% (v/v) Triton X-1, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM sodium ascorbate (pH 7.0) and incubated at 37°C

overnight for blue colour development. After staining, the plant material was thoroughly washed with 70 % (v/v) ethanol two or three times.

Molecular characterization of transgenic plants

Leaf tissues of transformed plants along with control plant leaves were used for the isolation of total RNA using the TRIzol method (Sambrook *et al*., 1989). The quality and concentration of RNA were analyzed using RNA gel electrophoresis and Nanodrop (Thermo Fisher Scientific Company, USA). All RNA samples were stored at -80 °C until required. RNA samples (1000ng) were taken for cDNA conversion using Revertaid First strand cDNA synthesis kit (Thermo Fisher Scientific Company, USA). Integration of transgene was confirmed by polymerase chain reaction (PCR) using *GUS Plus* specific primers (GUSF1-5'-GTGGATGAGGAAGGCAAAGTGGTC 3' and GUSR1-5'-CCATCGAAGTACCATCCGTTATAG 3') as well as hygromycin (*HptII*) specific primers. The *HptII* gene forward and reverse primer sequences were 5'-TCCTGCAAGCTCCGGATGCCTC-3' and 5'-CGTGCACAGGGTGTCACGTTGC-3'. The PCR reaction mixture for both primers consisted of 2µl of cDNA, 12.5 µl of 2x master mix, 1.0 µl of primers in a 25 µl of volume. The denaturation step was at 94˚C for 6 min and 40 seconds, the annealing step at 57˚C for 40 seconds, and the extension step at 72˚C for 40 seconds. The PCR reaction was carried out for 35 cycles, with a final extension at 72˚C for 10 min, and eventually maintained at 4˚C. The same protocol was used for both genes. The expected PCR products were separated on a 1.0% (w/v) agarose gel and visualized under a UV spectrophotometer.

Results and Discussion

Effect of explant Source

The selection of explant wasa critical factor in genetic transformation studies due to the leaching of phenolic compounds, tannins, and flavonoids as well as its ability for regeneration. The activated charcoal was gradually decreasing the phenolic exudation as well as brown exudate accumulation in the medium. The explant was excited into small pieces by a surgical blade on the Petri plate containing citric acid and ascorbic acid solution to remove phenol content. The leaf explant had scraped using cellophanetape due to the presence of wax cuticles on the leaf surface which ensures easy transformation. In addition, the transformation efficiency was observed more in the basal leaf compared to the middle and apical leaf segments. Leaf explants are used mostly compared to other explants for *Agrobacterium*-mediated genetic transformation (Petri *et al*., 2008).

Bacterial Transformation

Among six different growth phases (OD_{600}=0.8, 0.7, 0.6, 0.5, 0.4, and 0.3), a late-log phase of 0.8 OD_{600} value was the most suitable density for plant transformation and produced the highest number of *GUS* positive explants. The optical density at the wavelength of OD_{600} nm 0.4 and 0.3 was not suitable for Transformation studies due to the minimum bacterial growth. Tissue damage occurred at optical density values greater than 0.8 because of bacterial overgrowth. An OD of 0.8 was the most effective for obtaining high rates of transformation in *Jatropha curcas* (Franco *et al*., 2016), Vanda Kasem's Delight Tom Boykin (VKD) orchid (Gnasekaran *et al*., 2014), and *Veratrum dahuricum* (Rui Ma *et al.,* 2020).

Plant Transformation

Wounding effect

From the wounding site, various phenolic substances were released and which enhanced the *vir* gene activation (Stachel *et al*., 1985, Gelvin and Stanton, 2000). The wound had been created using a surgical blade and forceps in the abaxial surface of leaf explants. The maximum transformation efficiency was recorded using wounded leaf explants compared to non-wounded leaf explants.It was also confirmed in the case of *J. curcas* (He *et al*., 2009) and *Cucurbita* species (Nanasatoa *et al*., 2013).

Infection and Co-Culture Duration

The infection duration played animportant role in the plant transformation system. The infection period of 30 min was optimum to transfer the *Gus Plus* gene into the explant and the maximum transformation efficiency was observed with 30 min long immersion. When the infection time exceed 30 min, it was not appropriate for gene transformation due to over-bacterial growth. A similar report was recorded in *Pelargonium graveolens* (Singh *et al*., 2016), and *Dieramae rectum* (Baskaran *et al*., 2017). The co-cultivation duration was an important factor that influenced gene transfer in the *Agrobacterium*-mediated system and it was carried out in dark at 28 °C. Mostly 2–7 days were considered for the co-cultivation period (Kumar *et al*., 2004). Three days of the co-cultivation period showed the optimum transformation frequency in this study whereas four days co-cultivation period resulted in bacterial overgrowth. A similar report had been shown in *Citrus limonia* (Almeida *et al*., 2003). The leaf explants remained stable after 14 days of incubation on selection media (Figure-2b), but all of the stem explants had dried up. This could be because stem explants that had been infected with *Agrobacterium* were unable to acquire T-DNA (Figure-2d).

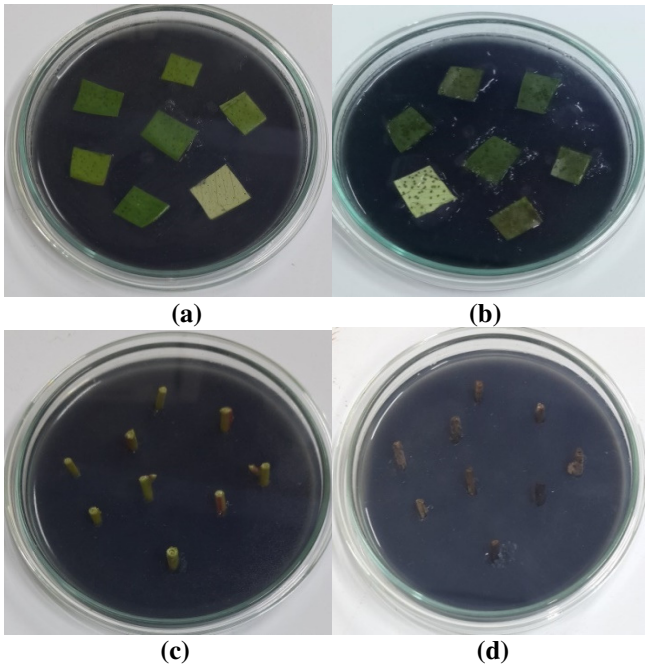
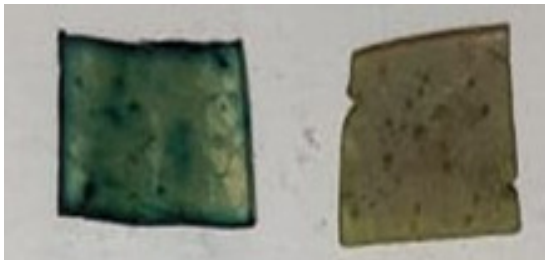


Fig. 2 : The explant of *S. glauca* in the co-cultivation medium. (a) Leaf explant, (b) Culture of inoculated leaf explants on charcoal medium. (c) Stem explant and (d) Dried stem explant.

Confirmation of transgenic plants

GUS assay of putatively transformed leaf explants:

Gus's expression was observed under the light microscope in transformed leaves after being stained with X-gluc. The appearance of the blue colour indicated the presence of the *Gus plus* gene, whereas the absence of the blue colour indicated the absence of the *Gus plus* gene shown in (Figure-3).

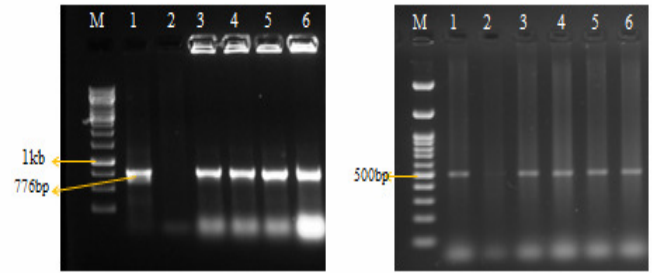


(a) **(b)**

Fig. 3 : *Gus* staining of *S. glauca*(a) Transformed leaf explant (b) Untransformed leaf explant

Molecular characterization of transformed plants:

The extracted cDNA samples were subjected to PCR analysis as rapid identification and the single band of 776bp(Figure-4a) and 500bp (Figure-4b) were amplified from four selected transgenic explants. But no amplification occurred in the non-transformed plant shown in (Figure-4).



(a) **(b)**

Fig. 4 : Molecular Confirmation of *Gus plus* gene in transgenic plantlets.

(a) M: 1kb Ladder; Lane 1- Positive Control; Lane 2- Untransformed explant (Negative Control); Lane 3 to 6: Single band of 776 bp which confirmed the presence of *Gus plus* gene.

(b) M: 100bp Ladder; Lane 1- Positive Control; Lane 2- Untransformed explants (Negative Control); Lane 3 to 6: Single band of 500 bp which confirmed the presence of the *HptII* gene.

Conclusion

This study emphasizes standardizing a strong protocol for *Agrobacterium*-mediated genetic transformation in *S. glauca*. The study has also determined that surface sterilization of explant, optical density, and co-cultivation period influenced T-DNA delivery. After being infected with *A. tumefaciens*, the transformed explants were maintained on MS medium supplemented with 3 mg/L 6-benzyl amino purine (BA), 0.01 mg/L indole-3-butyric acid (IBA), and activated charcoal. It was noticed that transformation efficiency was increased by wounding at the basal side of leaf explants and scraping wax off the leaves which enabled transformation efficiency. 0.8 OD_{600} value was found to be most suitable for plant transformation and 30 min of infection duration, as well as three-day co-cultivation, also played an important role in the gene transformation of *S. glauca*. PCR analysis proved the presence of the *Gus plus* gene and *HptII* gene in co-cultivated explants of *S. glauca*. It was further proven by *GUS* assay. This was the first study on establishing a stable protocol for stable genetic transformation in *S. glauca* using *A. tumefaciens*, which helps in enhancing the production of high medicinal value and biodiesel-producing *S. glauca*. This study also gave a way in establishing a strong protocol for stable genetic transformation and enhanced abiotic stress-tolerant tree species of high medicinal value and/or plants with increased biodiesel production.

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