International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 16-11-2021, Accepted: 02-12-2021, Published: 17-12-2021
Volume 6, Issue 6, 2021, Page No. 1196-1201

A simple and efficient *Agrobacterium*-mediated gene transformation in leaf explant of *Coleus forskohlii (Briq)*-GUS as a reporter gene

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Abstract
An efficient selection and plant rejuvenation protocol for *Agrobacterium* mediated gene transformation using leaf explant of *Coleus forskohlii* has been developed. Leaf explants from 30 days old *in vitro* plant were inoculated with *EHA*105 bacterial strain (carrying *NPT* II and *GUS* gene).Among the tested factor T-DAN transfer of plants, the EHA105 strain and addition of the Aceto-syringone to the co-cultivation medium increased the transformation efficiency. The factors affecting the frequency of transient gene expression were optimized, including leaf age, *Agrobacterium* concentration, infection time, Kanamycin concentration and co-cultivation period. The highest efficiency (32%) was obtained in 4 day pre-culture, 1.0 OD bacterial suspension culture, were sub-cultured on selection medium (MS medium with KIN 1mg/l + NAA 0.1mg/l +Kanamycin 50mg/l) after 3-day co-culture with Agrobacterium. Kanamycin levels above 50mg/l completely inhibited growth of untransformed region of the plants. The integration of selectable marker gene (*NPT*II) and reporter gene (*GUS* gene) into the genome of transgenic plants was confirmed using histoenzymatic *GUS* assay and polymerase chain reaction (PCR) respectively. These results pave the way for the transformation of *Coleus forskohlii* with desirable genes and should provide novel opportunity for the genetic improvement of more production of secondary metabolite in medicinally important of *C.forskohlii*.

Keywords: *Coleus forskohlii*, *Agrobacterium tumefaciens*, β-glucuronidase, transformation, *NPT* II gene

Introduction
Coleus forskohlii Briq. (Lamiaceae) is an important plant in Indian Ayurvedic medicine. It produces the labdane diterpenoid forskolin in its tuberous roots (Bhat *et al*. 1977) Forskolin effects heart action (positive inotropic effect), lowers blood and intraocular pressure and has anti-inflammatory properties. The sole source of forskolin is still the roots of wild or cultivated *C. forskohlii* plants. The general aim of the genome manipulation domain in such plants is to improve the methods for gene of interest transfer into the plant genome in order to improve the biosynthetic amount of the importance compounds (Natalia *et al*. 2002) [12]. In recent years, Agrobacterium mediated plant transformation, due to its simplicity and efficiency, has become quite often used method for the introduction of foreign genes into plant cells incorporated into chromosomes, and expressed in cells followed by regeneration of genetically superior plants. Transfer of DNA into plant cells via Agrobacterium, biolistic and other physical methods is now routine (Han *et al*., 1996; Kim *et al*., 1997) [11, 10]. Compared to direct DNA-transfer methods, the Agrobacterium-mediated transformation method is deliberated to be simpler and less expensive, as it activities the unique aptitude of Agrobacterium to introduce the transgene into plant cells. Separate micro projectile bombardment method, the most generally used direct DNA-transfer method, Agrobacterium-mediated transformation, does not necessitate particular apparatus (gene gun) or expensive consumables such as gold elements. Its main disadvantage is its prospective host range restriction. The later problem can often be overcome by improving tissue culture techniques, and choosing alternate selection methods as well as alternate Agrobacterium helper stains (Songstad *et al*., 1990) [15]. One of the requirements for successful plant transformation, is the availability of a regeneration protocol that is compatible with the gene transfer (Suma *et al*., 2008) [19]. Plant transformation vectors and procedures have been upgraded to increase the efficiency of plant transformation and to succeed unwavering expression of transgenes in plants. Developing protocols for effective genetic transformation of medicinal plants with distinctive metabolic pathways, is significant to understand the molecular basis and regulation of secondary metabolites in plants and to engineer them for specific metabolites.

The transformation method was successful in a sum of crops but it is still limited in therapeutic plants (Wang *et al*., 1997) [22]. One of the most appropriate methods for medicinal plants engineering is genetic transformation leading to increased synthesis of biological active substances in regenerated plantlets. Transformation efficiency of any plant is influenced by several factors, including Agrobacterium strain, addition of phenolic compounds (e.g.,acetosyringone) in the co-cultivation medium, wounding treatment of the target tissue (Godwin *et al*., 1991) [3] and appropriate selection of transformed cells or tissue from majority of untransformed tissue. The analysis of transformed plants can be confirmed by several methods using the applications of reporter genes like β-glucuronidase (*GUS*), green fluorescent protein(*GFP*), chloramphenicol acetyl transferase (CAT) and Discosoma sp. Red fluorescent protein (DsRed) (Wang and Xu, 2008; Chaofu and Jinling, 2008). The most commonly used visual reporter genes are *GUS a*nd *GFP* (Jefferson *et al*., 1987; Davis and Vierstra, 1998; Taylor and Fuquet, 2002) [9].

Glucuronidase is an enzyme that splits glucuronic acid molecules from other molecules by cutting glycosidic bonds. The enzyme exhibits both endo- and exo-glycosidase activities, in the sense that it can cleave monosaccharides at the middle or the end of the chain. In molecular biology, *E.coli* β-glucuronidase encoding gene, i.e., *GUS*, is used as a reporter gene to monitor gene expression, study promoter controlling elements and to detect tissue specific expression (Jefferson, 1987 & 1989; Jefferson and Wilson 1990; Jefferson, 1992) [9, 6, 8]. Likewise, β-glucuronidase enzyme activity is working to study the transient and constant plant transformation for a several applications (Serres *et al*., 1997). This study illustrates the possibilities of biotechnology for genetic transformation aiming at stimulation of secondary metabolite (forskolin) production in *in vitro* condition. This study was to develop a reliable transformation and regeneration system that could be effectively used for genetic improvement of *C. forskohlii*. We have established the effective and highly reproducible protocol for *Agrobacterium tumefaciens*- mediated transformation for regeneration of transgenic *C. forskohlii* plants.

Materials and Methods

Agrobacterium tumefactions mediated transformation

30days old *In vitro* propagated *C. forskohlii* plants leaf discs (9 mm diameter) were cut using a cork-borer. Leaf disc were pre-incubated on MS-shoot induction medium with KIN 1mg/l + NAA 0.1mg/l for 0-4ays with the abaxial side facing up. The appropriate *A. tumefaciens* stored at -80ºC were first activated by inoculation on solid YEM media and cultured at 28ºC for two days. Then the bacteria were transferred into liquid YEM media by using a sterile bacterial loop and cultured on an orbital shaker at 280 rpm and 28ºC until the culture reaches to 1 O.D at 600 nm. The culture was transferred to a sterile polypropylene tube and centrifuged at 5,000 rpm for 10 min and re-suspended with equal of liquid MS medium medium buffered with 12.5 mM sodium phosphate buffer (pH 5.6) (Sunilkumar *et al*., 1999) + 100 µM acetosyringone. The 30 days old *in vitro* explants were immersed in prepared bacterial suspension and swirled for various times (5, 10, 20 & 30 min) in different experiments. Infected explants were blotted on Whatman No. 1 paper to remove excess of Agrobacterium culture and placed directly on co-cultivation medium. Co-ultivation was carried out for different days at (1-4 days) light/dark condition. After the co-cultivated explants were washed with liquid MS shoot induction medium + 250 mg/l cefotaxime + 100 mg/l Kanamycin. Then, the explants were transferred to MS-shoot induction medium + 250 mg/l cefotaxime with different concentration (0, 25, 50, 100,125, and 150) of Kanamycin and placed in 16/8 h light/dark condition. After three weeks of initial selection, preformed shoots were cut off and rest of explant with shoot buds was placed on fresh MS shoot induction medium + 250 mg/l cefotaxime + 50 mg/l Kanamycin till further proliferation of shoot initials (10 to 25 days). Elongated shoots of 2 to 3 cm height were separated and transferred to MS rooting medium + 250 mg/l cefotaxime + 50 mg/l Kanamycin for root initiation. From the rooted plantlets segments were subjected to GUS analysis and other transgenic plants were hardened in greenhouse by transferring to pots.

Histoenzymatic GUS Assay

The histoenzymatic analysis of GUS activity in the transformed callus, leaf and shoot explants was performed according to Jefferson *et al*. (1987) [9]. The explants were incubated overnight at 37^{0}C with 0.1mM 5-bromo- 4-chloro-3indolyl β-D-glucuronide (X-gluc) in 50mM sodium phosphate buffer (pH 7) supplemented with 0.1 M Potassium Ferrocyanide,0.1M Potassium Ferricyanide and 10% W/V Triton –X-100.The GUS reaction was stopped by rinsing with 70% ethanol until pigments, such as chlorophyll, cleared completely. The number of individual cells of cell aggregates that contained a blue color was counted as expression units.

Polymerase chain reaction

Genomic DNA was isolated from young leaves of transgenic plants using C-TAB method (Doyle and Doyle 1987). The integration of the *NPT*II gene was confirmed by PCR using the primer. The *NPT*II gene was amplified by polymerase chain reaction (PCR) using the 5'-TCGGCTATGGGGCACAACAGA-3' and 5'-AAGAAGGCGATAGAAGGCGATGCG- 3' primers that amplify a 700-bp fragment corresponding to the coding region of the *NPT*II gene. The transformed and non-transformed plants (control) and water control (W) were used as negative and environmental controls, respectively. PCR amplification was carried out in a thermal cycler (Eppendorf, Germany) programmed with an initial denaturation of DNA at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, an annealing step at 60 °C for 1min and 72 °C for 1min, and a final extension step at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on 1.0 % agarose gel.

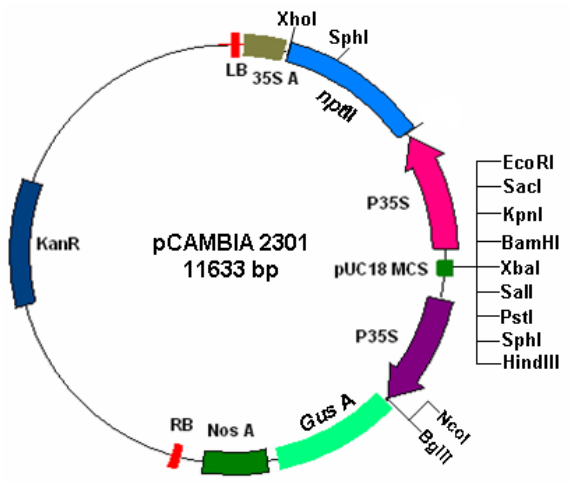

Fig 1: pCAMBIA2301 binary plasmid circular map

pCAMBIA 2301 as binary plasmid carrying NPTII gene as plant selection marker under CaMV 35S promoter and terminator & GUS A as reporter gene under Ca MV 35S promoter and Nos terminator.

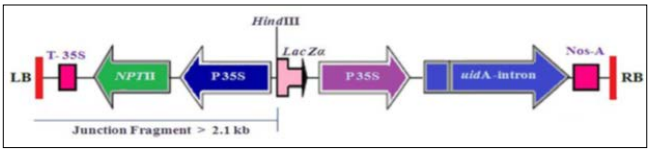

Fig 2: Linear diagram of T-DNA portion of pCAMBIA2301 binary plasmid carrying *NPT*II and *GUS* cassettes

Schematic representation of the T-DNA (5.2 kb) of pCAMBIA2301 binary vector containing the *uidA* and *NPT*II genes. The position of *Hind*III is indicated on the T-DNA. No other *Hind*III sites are present on pCAMBIA2301 (total size 11.6 kb). LB/RB: left and right T-DNA border sequences

Results and Discussion

Determination of suitable Kanamycin concentration in selection medium

In the current study, *In vitro* selection of resistant explants against kanamycin was carried out at a concentration of 0-150 mg/l. There was no considerable effect of kanamycin on shoot regeneration when used at a concentration of 0-25 mg L-1. The regeneration frequency decreased quickly when kanamycin concentration was increased (Fig-3). Approximately 53% shoots survived at concentrations of 50 mg/l. At a concentration of 75mg/l and 100 mg/l, explants continued green for initial after days turned brown and decreased explant growth. The kanamycin concentration of 125mg/l and 150mg/l were used to inhibit 100% explants growth. The commonly used selection system, with kanamycin as selective agent and *NPT*II gene as selectable marker, was used in the present study for early identification and selection of transformed plants. Kanamycin at 50 mg/L neither necrosis the explants nor impaired normal shoot regeneration and shoot vigour but bleached the emerging shoots from non-transgenic cells. In a previous study of the effects of antibiotics on *C.forskohlii* in transformation studies (Neha Guleria *et al.*, 2015), the kanamycin was found to be a suitable selective agent. These results into concern, to reduce escape and prevent necrosis, 50 mg/l concentration of kanamycin were used for the selection and regeneration of *C. forskholii* plants with GUS gene.

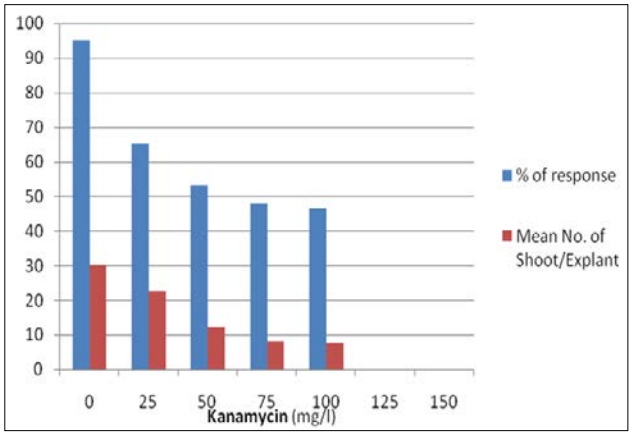

Fig 3: The effect of selection marker Kanamycin sensitivity

Influence of pre-culture and addition of Acetosyringone in Co-cultivation

Explants excised from young leaves (10-25 days old) from *in vitro* plants of *Coleus forskohlii* were tested for their suitability to achieve higher transformation. While we identified no significant variances among pre-culture stages of different intervals (0, 2, 3, and 4 days) based on transient *GUS* expression, a 4-day pre-culture suggestively increased the number of leaf explants able to grow under kanamycin selection (Table-1). Four days of pre-culture enhanced the retrieval of kanamycin-resistant explants several-fold. Since host cell division is essential for effective *Agrobacterium* transformation, it is not surprising that pre-culture in a high auxin medium frequently increases transformation percentage (Binns and Thomashow 1988) [1]. Transformation studies in other plant species have indicated that acetosyringone at an appropriate concentration enhances efficiency of transformation (Srivastava *et al*., 2009) [16]. Acetosyringone is one of the phenolic compounds secreted by wounded plant tissue and is known to be an effective inducer of Agrobacterium vir genes (Stachel *et al*., 1985) [17]. Numerous reports suggest that acetosyringone pre-induction of Agrobacterium and/or inclusion of acetosyringone in the co-cultivation medium can improve significantly Agrobacterium mediated transformation (Yao, 2002; Sunikumar *et al*., 1999) [20]. In our investigate; acetosyringone was included at a final concentration of 100 µM during the final stage of Agrobacterium growth and during co-cultivation. acetosyringone at 100 µM was found to give the best regeneration response. Transformation studies in other plant species have indicated that acetosyringone at an appropriate concentration enhances efficiency of transformation (Srivastava *et al*., 2009) [16].

The number of *GUS* positive explants was higher when acetosyringone was included in the medium. The results suggest that acetosyringone can be used to achieve significant improvements in transformation of *C. forskohlii*. During co-cultivation of the explants with Agrobacterium, growth of *Agrobacterium* on the explants should be avoided to prevent the turning necrotic or renewing few transgenic plants. The delay in exposing the explants to the selective negotiator kanamycin is acute in promoting regeneration and recovery of transgenic cells that give increase to transgenic plants. Correspondingly, extended infection time could also lead to necrotic explants resulting in failure of transformation (Table-1).

Production of putative transgenic plants

Explants after transformation were regenerated on SIM (shoot bud induction medium- Kinetin 1mg/l +NAA 0.1mg/l) which contained 50 mg/l Kanamycin and 250 mg/l Cefotaxime. High frequency transformation using *A. tumefaciens* (*EHA* 105 carrying pCAMBIA 2301) depends not only on the efficacy of the plant *in vitro* redevelopment system but also on the ensuing elimination of bacterial cells from transformed plant tissues. As the continuous presence of *A. tumefaciens* affects with the growth and increase of transformed cells and causes the death of the cultures, the elimination of *A. tumefaciens* completed by adding of antibiotics is an imperative stage for effective transformation. Selection during shoot initiation and shoot out-growth encourages rejuvenation of putative transgenic shoots. However, the quantity of selective negotiator used in the medium during these stages should not be too high, consequently the selective negotiator (Kanamycin & Cefataxime) concentration was reduced into half and redeveloping explants were transferred to fresh medium every 15days to continue the selection compression.

Table 1: Influence of various factors in *A. tumefaciens* mediated transformation in *C.forskohlii*

Factors	Treatment	No of explant	Number of Kanamycin (50mg/l) resistant shoots (%)	Gus^{+} Shoots
Pre-culture period	0	25	-	-
	2 day	25	4 (16)	1
	3 day	25	5 (20)	3
	4 day	25	7 (28)	6
Incubation period with agrobacterium	5 min	25	3 (12)	1
	10 min	25	7 (28)	4
	20 min	25	9 (36)	7
	30 min	25	6 (24)	2
Co-culture period	1day	25	2 (8)	1
	2day	25	5 (20)	2
	3 day	25	8 (32)	6
	4day	25	6 (24)	2
	5day	25	3 (12)	1

Fig 4: *Agrobacterium tumefaciens* mediated genetic transformation of *C. forskohlii* using *pCAMBIA2301* binary vector

a -Coleus forskohlii -Mother plant in in vitro culture
b- Pre-incubation and Co –Cultivation of Leaf explant(MS media with Kanamycin (50mg/l)
c & d-Callus and shoot development in transformed leaf explant on Kanamycin media
e- Multiple shoot formation
f- Transformed plantlets
g & h –Hardening of transgenic plants

GUS Assay

Th Histoenzymatic GUS assay was achieved by overnight incubation of putatively transformed *C. forskohlii* callus, leaf and shoots to examine the expression of *GUS* gene. GUS positive events or β-glucuronidase activity (Blue color) was observed on the calli, leaf and shoots which indicate the expression of *GUS* gene (Fig-5 a,b,d,e & g). Whereas, control calli leaf and shoots found to be *GUS*

negative (Fig-5 c,f & h). The intensity of GUS staining was higher in callus and leaf region. The explants were sampled after 4 weeks of culture on selection medium. Transformation efficiency was found to be 25%. The *Escherichia coli* gene *GUS* A encoding b-glucuronidase (GUS, Jefferson *et al*., 1987) [9] is commonly used as a reporter in constructs for plant transformation because there is almost no endogenous GUS activity in most plant species, its activity can be analyzed histochemically and easily quantified using fluorescent substrates (Quaedvlieg *et al*., 1998).

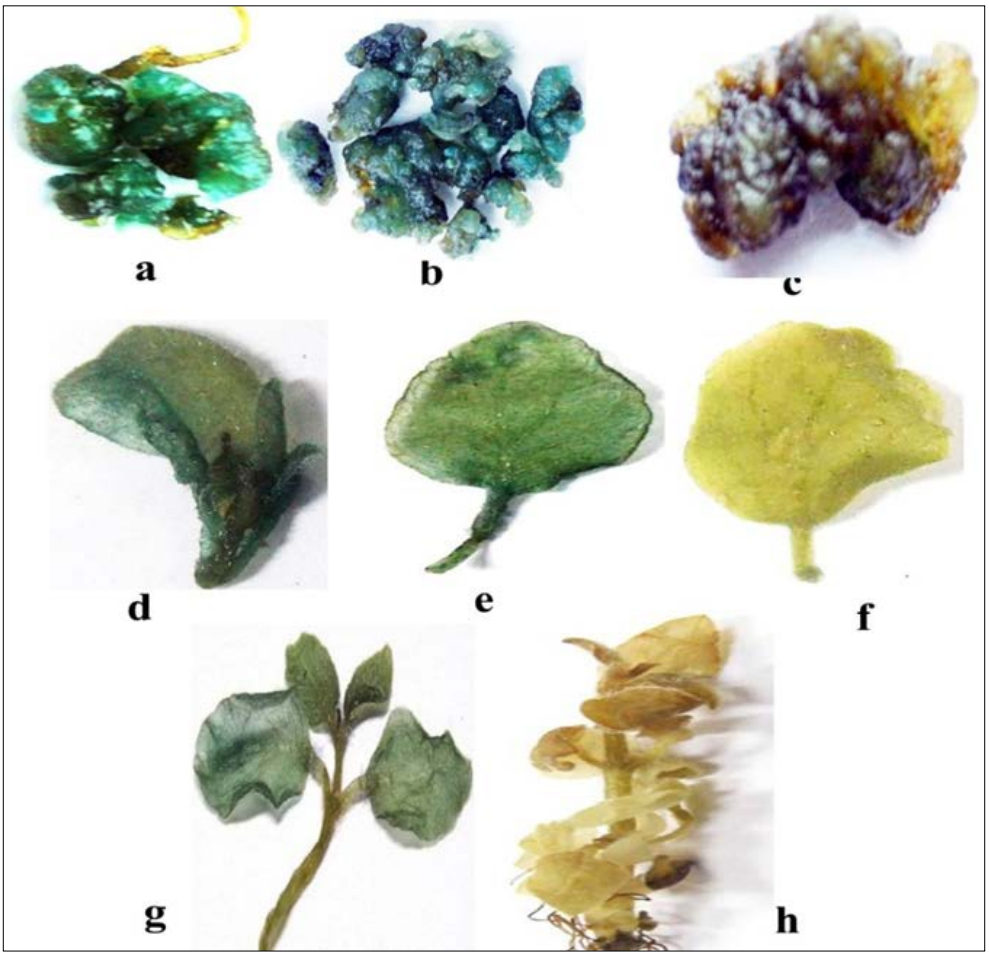

Fig 5: GUS gene expression in *C. forskohlii*

a, b- gene expression (blue color formation) in Transformed callus
c- Untransformed callus (No blue color formation)
d, e& g- gene expression(blue color formation) in transformed leaves and shoot
f & h- untransformed leaf and shoot(No blue color formation)

PCR Analysis

Molecular analysis was carried out through Polymerase hain Reaction (PCR). DNA isolated from putative transgenic plants and control plant. The amplification was confirmed by the presence of *NPT*II gene in kanamycin resistant putative transformed plants that was achieved after the selection, Co-cultivated with *EHA*105 *Agrobacterium tumefaciens* strain harbouring the binary plasmid pCAMBIA2301. The gene specific primers for *NPT*II gene (Fig-6) were used for PCR amplification. The presence of a band at 700 bp in samples from transformed plants (Fig-6 lanes1, 2, 3, 4, 5, & 6) confirmed the integration of the *NPT*II gene. Amplification of this fragment (700 bp) was not observed in control plants (Fig-6 c) the transformation of plants by *Agrobacterium* is a multi-faceted process that involves various steps and the determined action of both microbial and host factors. *Agrobacterium tumefaciens*-mediated gene transfer is influenced by strain (Stomp *et al.*, 1990), explant type (Humara *et al.*, 1999) [4], temperature (Dillen *et al.*, 1997) [2], size of T-DNA (Park *et al.*, 2000) [14], and constitutive expression of the virulence genes (Hansen *et al.*, 1994; Rossi *et al.*, 1996) [11]. In total, from the bacterial point of view, for best transformation efficiency, a slightly stressed state of host cells appears desirable. Commonly, plants may avoid *Agrobacterium* infection if their defense system is in a warned state

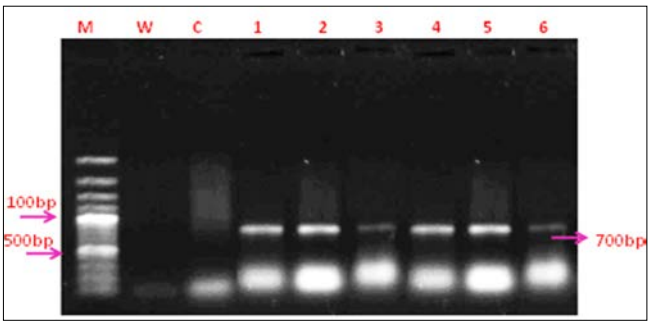

Fig 6: PCR analysis of putative transgenic *C.forskohlii* plants carrying *pCAMBIA2301* binary plasmid with *NPT*II gene specific primer

lane M- 1kb DNA Ladder
lane W- Water Control
lane C- Control (Untransformed Plant sample)
Lane L1 to L6 - Transformed Plant sample

Conclusion

To summarize, we have standardized an efficient transformation protocol for a *C. forskohlii* and optimized the antibiotic concentration. Integration of exogenous gene into the genome of *C. forskohlii* was confirmed by GUS histoenzymatic assay and PCR analysis. The super virulent strain of *Agrobacterium tumefaciens EHA*105 (pCAMBIA2301) was able to transfer and insert a T-DNA containing *NPT*II and GUS genes into the regeneration competent cells present at the entire leaf explants.The leaf explants produced the maximum number of shoots per explant, and the redevelopment site was entirely exposed and simply available to *Agrobacterium*. This simple transformation method could be used for learning gene manipulation and for transferring different characters into *C. forskohlii*.

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