International Journal of Agriculture, Environment and Biotechnology
Citation: IJAEB: 8(2): 227-235 June 2015
DOI Number: 10.5958/2230-732X.2015.00029.7

GENETICS

An Efficient Regeneration and Genetic Transformation Protocol of *Coleus forskohlii* using Biolistic Gun

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Paper No. 313 Received: 20 March 2014 Accepted: 22 April 2015 Published: 28 June 2015

Abstract

An efficient selection and plant regeneration protocol for biolistic gun transformation using leaf derived callus of *Coleus forskohlii* has been developed. Highest regeneration frequency 90% with 50 shoots per callus clump was obtained on Murashige and Skoog (MS) media supplemented with benzylaminopurine (BAP) 2.0 mg L^{-1} + naphthalene acetic acid (NAA) 0.5 mg L^{-1} The rate of shoot multiplication was increased with each subculture. Rhizogenesis was obtained on the same media composition. The *in vitro* raised plants were established successfully in sand and cocopeat (1:1). Callus of *C. forskohlii* was bombarded using biolistic gun with pABC plasmid DNA which contains β-glucuronidase *(GUS)* reporter gene and *Arabidopsis thaliana* white brown complex homologs (*AtWBC19)* as selectable marker gene. Kanamycin in the shoot induction medium was compared qualitatively and quantitatively for its efficiency as a selection agent for the selection and regeneration of transgenic plants after biolistic gun transformation. Kanamycin levels at or above 50mg L^{-1} completely inhibited growth of untransformed shoots. The integration of selectable marker gene *GUS* and *AtWBC 19* 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.

Highlights

- Regeneration and Biolistic gun transformation protocol was standardized for *Coleus forskohlii.*
- *GUS* and *AtWBC 19* gene integration was confirmed using GUS assay and PCR.

Keywords: *AtWBC 19*, biolistic gun, *Coleus forskohlii*, genetic transformation, *GUS*

In vitro regeneration, genetic transformation combined with molecular biology has become major biotechnological tools used for multiplication, genetic enhancement and molecular characterization of medicinal plants. The success in this domain represents the culmination of many years of efforts in micropropagation and improvement of plant genetic engineering techniques (Hansen *et al.* 1999). Over the past decade, plant cells have opened new advances for the production of natural or recombinant compounds of commercial importance (Xu *et al.* 2012). The expression of subunit vaccine antigens in plants has emerged as a convenient, safe and potentially economical platform technology (Thomas *et al.* 2011). Advances in transgenic technology bring new responsibilities for safe use of transgenic plants for the benefit of humanity and the environment (Fischer *et al.* 2012). The number of factors to be taken into consideration in the development of any transgenics. First and foremost is to recognize the plants that produce vaccines and should be grown, processed and regulated as pharmaceutical products.

Secondly, the potential for scale-up production to produce sufficient quantities, cost effectiveness and most importantly safety issues (Francesco *et al.* 2003).

An important aromatic herb in Indian Ayurvedic medicine considered endangered medicinal plant, the *C. forskohlii*. It belongs to the *Lamiaceae* family is valued for the production of labdane diterpenoid forskolin from its tuberous roots used for relief of cough, eczema, skin infections, heart failures and certain type of cancers (Boby and Bagyaraj 2003). The general aim of the genome manipulation domain in such plants is to develop techniques for gene of interest transfer into the plant genome in order to improve the biosynthetic rate of the interest compounds (Natalia *et al.* 2002). *C. forskohlii* is a potential candidate to produce vaccine as this crop is vegetatively propagated and can be grown in green house for trials without any chances of genetic contamination (does not set seeds), so it offers good platform to develop transgenics. By this means, very large quantities of recombinant protein can be produced quite rapidly, thereby significantly reducing the cost of production.

Biolistic gun is a commonly used method for genetic transformation of plants because of its physical nature and simple methodology. It can be used for either transient or stable transformation. Many "firsts" were achieved through the application of biolistic technology including chloroplast and mitochondria transformation, as well as nuclear transformation of important monocot species such as wheat, corn and rice (Sanford 2000). Efficient and stable transformation system based on gene gun are well established for tropane alkaloid-producing medicinal plant, *Hyoscyamus muticus*, and also in *Allium sativum* (Baratali *et al.* 2011). The relative simplicity of this method is laying the foundation for its future use in the production of genetically modified plants. The standardization of plant regeneration protocol is a pre-requisite for any successful plant genetic transformation. The use of optimum concentration of selection agent is also important. Without them, creation of transgenic crops is not feasible on purely economic and practical terms. These systems allow the relatively straightforward identification and selection of plants that have stably incorporated the marker genes along with the genes of interest (Narendra *et al.* 2012). To date, in plant transformation studies, most widely used selection agent is kanamycin including *C. forskohlii* transformation (Khin *et al.* 2006). To reduce the frequency of false positive and non-transgenic shoots it is necessary to optimize the antibiotic concentration for selection, as stringency of kanamycin selection for transgenic shoots is, however highly species dependent. Thus, the work described here was initiated to establish reliable protocol for plant regeneration of *C. forskohlii*, the best concentration of antibiotic (kanamycin) and genetic transformation using biolistic gun method. The present work is the first report on transformation studies of *C. forskohlii* using biolistic gun.

Materials and Methods

Plant material and regeneration

Apical young leaves were collected from greenhouse grown *C. forskohlii* plants and were used for plant regeneration and genetic transformation studies. The explants were kept under running tap water to remove adhering dust particles and surface sterilized with 0.8% (w/v) bavistin and 0.1% (w/v) mercuric chloride for 1-2 minutes, followed by 3-4 washing with sterile distilled water, under aseptic conditions. After four rinses in sterile distilled water, leaves were trimmed into pieces of about 1 cm^2 and then inoculated onto culture media prepared using Murashige and Skoog (1962) salts, added with MS micronutrients, vitamins, 3% (w/v) sucrose 0.8% agar and supplemented with different concentration of BAP (0.2 and 0.3 mg L^{-1}) along with NAA (0.4,0.5, 0.6, 0.7 and 0.8 mg L^{-1}). The pH of the media was adjusted to 5.8 with 0.1 N NaOH, autoclaved at 121°C and 16 pounds/square inch for 15 min. The cultures were maintained at 25 ± 2°C in the growth chamber under a 16 h photoperiod using white fluorescent tubes. All cultures were maintained for 4 weeks without subculture to fresh medium. Regenerated shoots were subcultured in every 15 days of interval

and rhizogenesis was obtained on same media. Regenerated plantlets with well-developed shoots and roots were washed under gentle flow of tap water for four hours to remove adhering agar completely. Plantlets were transferred to plastic pots filled with cocopeat and sand ratio (1:1). Plantlets were watered as per requirement and gradually acclimatized to field conditions. The acclimatized plants were transferred to normal bigger pots containing 2/3 normal soil and 1/3 cocopeat and kept under normal day length conditions in green house.

Determination of optimal Kanamycin concentration

Atwbc19 has been shown to be highly specific for kanamycin resistance in transgenic plants; that is, it does not confer resistance to geneticin, neomycin or other clinically used aminoglycoside antibiotics (Mentewab and Stewart 2005). Therefore, before transformation kanamycin sensitivity test was performed on explants. To determine the optimal concentration of kanamycin on callus formation and shoot regeneration, leaf explants were cultured on shoot induction media supplemented with different concentrations of kanamycin (0,5, 10, 20, 30, 40 ,50, 60, 70, 80, 90 and 100 mg L^{-1}). Kanamycin was filter sterilized and added to the cooled medium (< 45^0C) after autoclaving. All cultures were maintained at 25 ± 2°C in the growth chamber under a 16 h photoperiod using white fluorescent tubes. Each explant was scored for lethality after 15 days.

Biolistic gun Transformation and plant regeneration

For biolistic gun bombardment, the plasmid pABC which contains *GUS* reporter gene and a selectable marker gene *AtWBC19* that encodes specifically for kanamycin (Mentewab and Stewart 2005), both driven by modified CaMV 35S promoter, was used (Figure 1). Callus of *C. forskohlii* were arranged in a circle of about 3cm diameter in the centre of mannitol agar petri plates and maintained for 4 hours. Bombardment was carried out with a Biolistic PDS-100/He particle delivery system (Bio-Rad). All bombardments were performed at a pressure of 1100psi, a 9 cm particle travel distance, an 11 mm gap distance (between the rupture disc and the macrocarrier) and a vaccum of 25 Hg inch (Shripad *et al.* 2010). Following bombardment, explants were incubated in the dark for overnight at 26°C. Initially, callus explants were placed on shoot induction medium of BAP 2.0 mg L^{-1} and NAA 0.5 mg L^{-1} without any selection agent i.e. kanamycin. Two weeks later resistant explants were sub-cultured to fresh shoot induction medium containing kanamycin for selection. Rooted plants were hardened in green house by transferring to pots.

Histoenzymatic GUS Assay

The histoenzymatic analysis of GUS activity in the transformed callus explants was performed according to Jefferson *et al.* (1987). In brief explants were incubated overnight at 37^0C 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 *AtWBC19* gene was confirmed by PCR using the primer set 5′-ACTGCAGGTACCA TGAATCTATCACTCAGGGG-3′SacI (forward) and 5′- GTCAGCGAGCTCTCACGTCCTCTTATTCCT GC-3′ KpnI (reverse). The amplification was done using Rcorbett research Model CG1-96. The PCR programme was set at 94^0C for 5 min followed by 30 cycles of 94^0C for 1 min, 58^0C for 1 min and 72^0C for 1.5 min, with a final extension at 72^0C for 10 min.

Table 1. Effect of BAP and NAA on shoot regeneration from leaf explants of *C. forskohlii*

MS media with (mg l^{-1}) BAP	NAA	Frequency of callus regenerated into shoots (%)	No. of shoots per explant	Average No. of days taken for shoot initiation	Shooting observation	Average No. of days taken for rooting	Root number/ shoot*	Rooting observation
2.0	0.4	60.4^{c}	14.8^{b}	35	Short, healthy	20	5.2^{b}	Thin, longer
2.0	0.5	93.4^{a}	35^{a}	30	Long, Healthy	14	8.8^{a}	Thick, longer, healthy
2.0	0.6	57.8^{d}	10.2^{d}	42	Long, vitrified, weak	22	4.4^{c}	Thin, shorter
2.0	0.7	77.2^{b}	14.6^{c}	45	Short, not healthy	25	4.0^{d}	Thin, shorter
2.0	0.8	53^{e}	10^{e}	49	Short, vitrified	29	1.8^{e}	Long, thin

15 explants cultured per treatment

*Different letters within the column indicate significant differences according to LSD (P≤0.05) following ANOVA

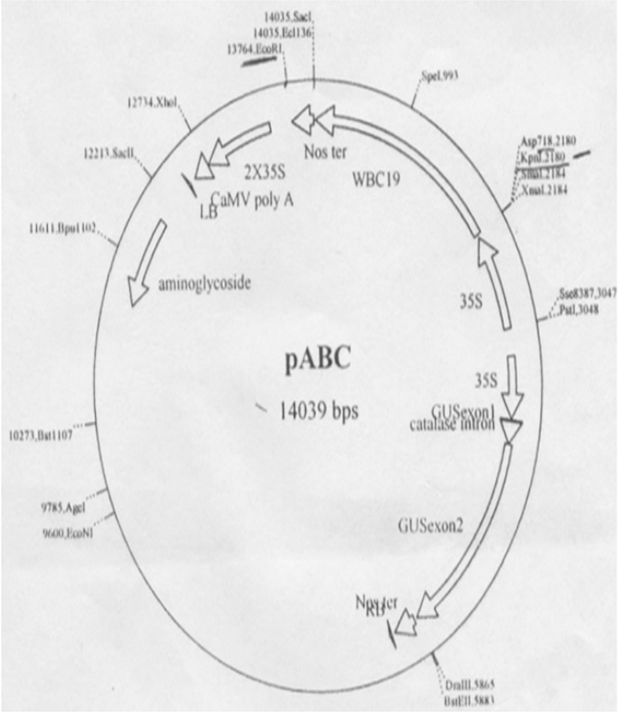

Figure 1: Vector map of plasmid (pABC) containing *AtWBC19* and *GUS* gene

Statistical analysis

Each treatment had 5 replicates with 15 explants and all the experiments repeated twice to see the reproducibility of the results. The data were reported as mean standard error and means were analysed by Analysis of variance (ANOVA) and significant differences between means were compared by least significant difference (LSD).

Results and Discussion

Plant regeneration by organogenesis

Surface sterilized, leaf segments of *C. forskohlii* were cultured on MS medium supplemented with different concentrations of BAP and NAA. After 15 days of culturing, callus initiation and shoot regeneration was observed from the margins, midrib and abaxial side of leaf explants. Repeated experiments revealed that highest regeneration frequency 90% with 50 shoots per callus clump was obtained with BAP 2.5 mg L^{-1} + NAA 0.5 mg L^{-1} (Figure 2a and Table 1). The rate of shoot multiplication was increased with each subculture. Rhizogenesis was obtained on the same media composition (Figure 2b). Over 90% of shoots formed roots after 15 days of shoots with average number of primary roots per shoot ranging from 5

to 8 (Table1). After acclimatization, rooted shoots were transplanted into pots and maintained in green house (Figure 2c and 2d). Percentage survival of hardened plants was found to be 70%.

Figure 2. Standardization of regeneration of *C. forskohlii* from leaf explant (a) Adventitious shoots (b) Well developed rooting system (c) Hardened regenerants (after 20 days of hardening) (d) after 35 days of hardening

Effect of Kanamycin concentration

In the present study, *In vitro* selection of resistant explants against kanamycin was carried out at a concentration of 5- 100 mg L^{-1}. There was no much effect of kanamycin on shoot regeneration when used at a concentration of 5-40 mg L^{-1}. The regeneration frequency decreased rapidly when kanamycin concentration was increased (Figure 3). Approximately 40% shoots survived at a concentrations of 50 mg L^{-1}. At a concentration of 55 mg L^{-1}, explants remained green for initial 5 days then turned brown. At 70 mg L^{-1} 90% of explants bleached and died. Further increase in the level of kanamycin to 90 and 100 mg L^{-1} totally inhibited callus induction. Taking these results into consideration, to minimize escape and prevent necrosis, 50 mg L^{-1} concentration of kanamycin was used for the selection and regeneration of *C. forskholii* plants with *GUS* gene.

Genetic transformation and regeneration of putative transgenic

To obtain regeneration of transgenic plants, the bombarded calli (Figure 4a) were cultured on a succession of selective media allowing bud formation (Figure 4b), shoot elongation and root formation. The putative transgenic plantlets regenerated were morphologically identical to non-transformed plants considered as control (Figure 4c and d), but their development and growth were slower. As a consequence of this delay, which was probably caused by kanamycin, plantlets were obtained only after two months. The survival percentage, calculated as a percentage of calli explants giving putative transgenic plantlets, and found to be 90%.

Molecular characterization of the integrated gene

Histoenzymatic GUS Assay

Histoenzymatic GUS assay was performed by overnight incubation of putatively transformed *C. forskohlii* callus to investigate the expression of *GUS* gene. GUS positive events or β-glucuronidase activity (Blue coloration) was observed on ten bombarded calli which indicate the expression of *GUS* gene. Whereas, 30 bombarded calli and control, found to be GUS negative (Figure 5). The calli of explants were sampled after 4 weeks of culture on selection medium. Transformation efficiency was found to be 25%.

Polymerase Chain Reaction Analysis

To confirm the integration of the *AtWBC19* selectable marker gene into the genome of putative transgenic plants, a PCR analysis was carried out. Using the *AtWBC19* primer set, the expected 2.1kb fragment was found in all GUS positive plants alongwith positive control (plasmid DNA pABC) but not in control plants (Figure 6). The amplification of DNA samples from GUS positive plants confirms the

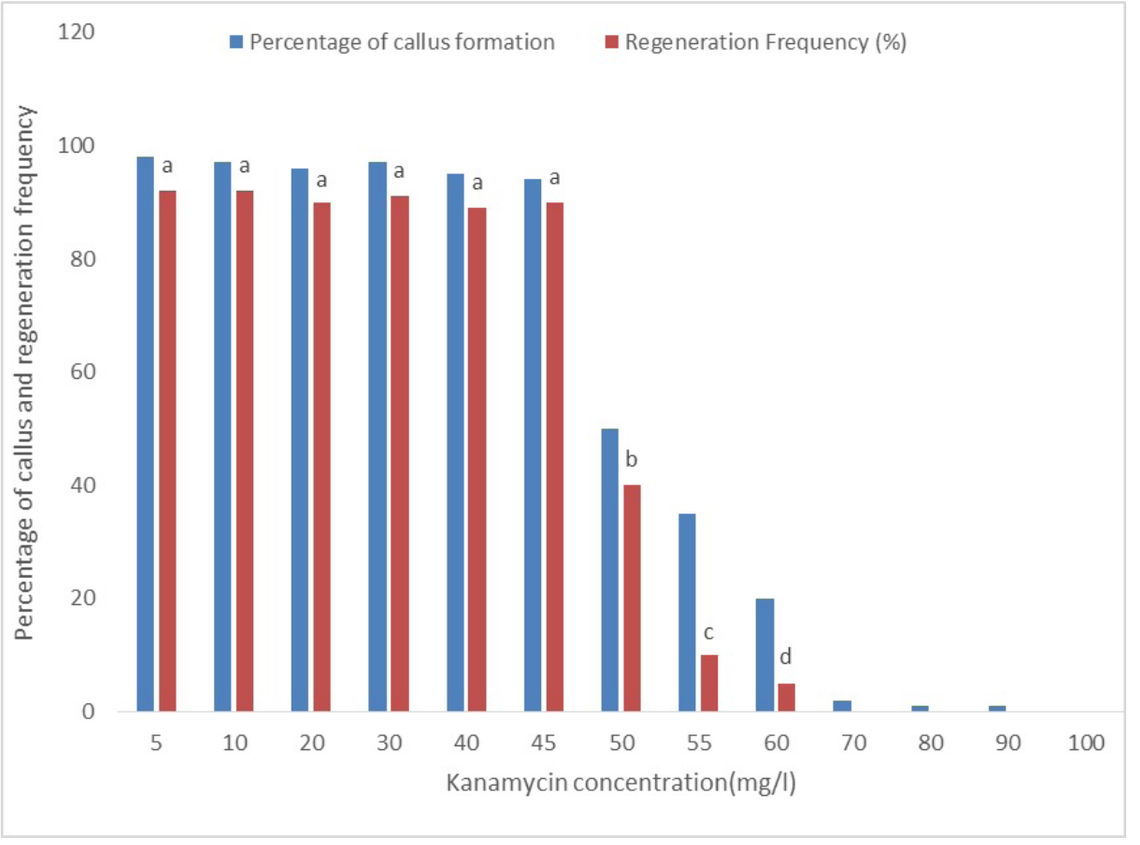

Figure 3: Effect of kanamycin concentrations on percentage survival of leaf explant of C. *forskohlii*. Twenty explants were taken for the experiment. Each experiment was repeated twice. Data represents mean values ± standard error (SE) of five replicates. Means with common letters are not significantly different according to LSD ($P \leq 0.05$) following ANOVA

integration of exogenous DNA into the plant nuclear genome.

In recent years, a mass propagation and reliable regeneration procedure for medicinal plants have been established, because it would be a primary step to facilitate gene introduction and transferring new traits (Siahsar *et al.* 2011). In the development of any regeneration protocol, growth hormone combination and choice of explant play a major role (Kaur *et al.* 2011). In the present study, combination of BAP 2.0 mg L^{-1} and NAA 0.5 mg L^{-1} proved to be the best. These combinations of growth hormones differ considerably from those previously reported by Prathiba *et al.* (2011) and Sharma *et al.* (1991) where 1.5 mg L^{-1} alone was optimal, and kinetin 2.0 mg L^{-1} and IAA 1.0 mg L^{-1} was found to be best combination when nodal explants of *C. forskohlii* were used for micropropagation, respectively. This could be possibly due to the use of different explants used in the studies. Therefore, type of explant also had a significant affect on shoot regeneration studies. However, they are consistent with data obtained earlier in our laboratory by Khin *et al.* (2006), Hajin *et al.* 2011, Bariya and Pandey 2014 showing that combination involving high concentration of cytokinin and low concentration of auxin promote adventitious shoot formation in *C. forskohlii Mentha aquatica* and Corn mint leaf explants respectively.

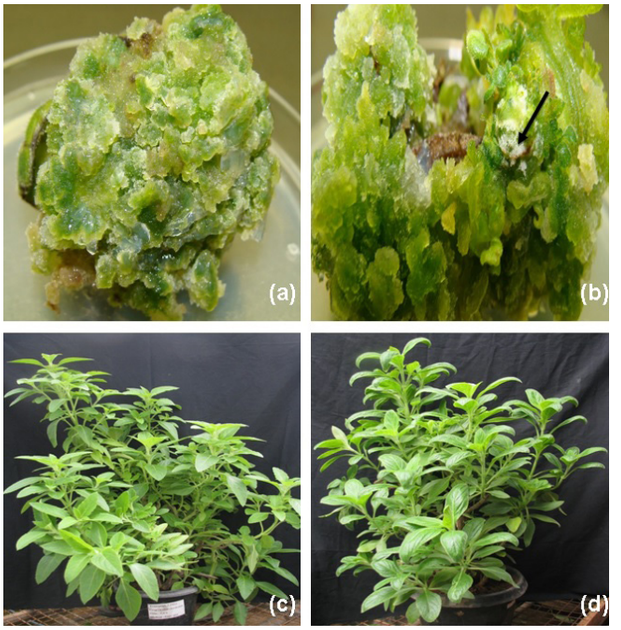

Figure 4. Development of transgenic plants of *C. forskohlii* (a) callus bombarded with pABC plasmid (b) kanamycin resistant callus showing bud (arrow) on regeneration medium containing 50mg L^{-1} kanamycin (c) transgenic plant of *C. forskohlii* after transformation (d) Control plant of *C. forskohlii*

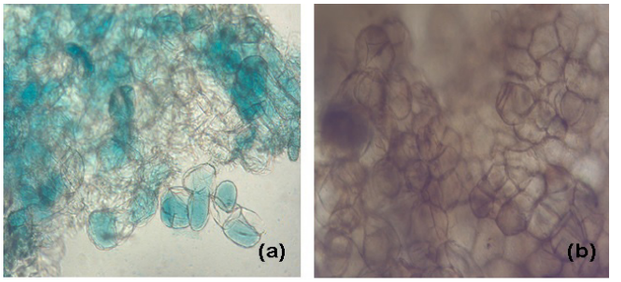

Figure 5. Histoenzymatic GUS Assay for Biolistic gun transformation; (a- GUS positive result by Transgenic plant; b- Control)

Similar effect of BAP and NAA was reported in *Thapsia gargangia* (Makunga *et al.* 2003) and *Jatropa curcas* (Verma 2013). Rooting in all the experiments were obtained when cultures kept for a longer time on same media. This may be due to the addition of NAA in regeneration media enhances the root formation. In tissue culture, cytokinin interact with auxin during the formation of plant organs such as shoots and roots (Ying *et al.* 2011).

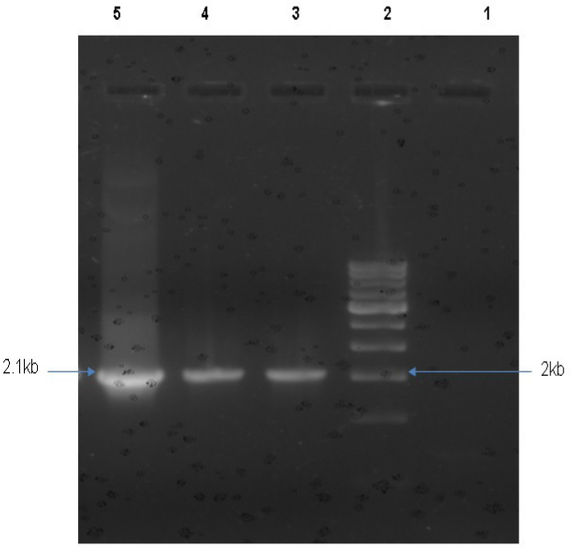

Figure 6. PCR analysis of DNA isolated from leaves of non-transformed (control) C. *forskohlii* and putative transgenic plants. Agarose gel electrophoresis of PCR amplification was performed with primers for the *AtWBC19* gene Lane 1: Non-transformed plant (control), Lane 2: Molecular size marker (1kb Genei), Lane 3, 4: Putative transgenic plants, Lane5: plasmid pABC (positive control)

In transformation studies, selectable marker genes play a major role in the selection of transgenic plants. *AtWBC19*, which confers specifically kanamycin resistance, used as an alternative to bacterial antibiotic resistance gene (Mentewab and Stewart 2005). As this marker is endogenous to and presumably ubiquitous in plants, its use in the selection of transgenic plants might be more widely accepted by the public and regulators and inherently less risky than bacteria-derived resistance markers (Kang *et al.* 2010). Optimization of antibiotic concentration is prior necessity to achieve stringent selection and confirmed transgenic plants. Optimized kanamycin concentration was found to be 50 mg L^{-1}. These results are in line with the study conducted by Chaithra 2014 and Khin *et al.* (2006), who studied transformation in *Solanum tuburosum* and *C. forskohlii* respectively. In contrast, the study done by Smrati and Shilpi, (2011) (unpublished) on transformation of *Ocimum* kanamycin concentration used was 30 mg L^{-1} and in *Mentha piperita* L. optimized concentration was found to be 15-20 mg L^{-1} (Krasnyanski *et al.* 1999)

this could be explained by the fact that selection and shoot regeneration is highly species and cultivar dependent.

Earlier study done in our laboratory on rice by Ramachandra *et al.* (2014) achieved maximum transformation efficiency when tissue was bombarded at 1100psi and target cell distance of 9cm. These results are in line with the present study. During GUS histoenzymatic assay blue coloration of calli confirmed the expression of *GUS* gene (Singer *et al.* 2011). Similar *GUS* gene expression pattern was observed previously for *Citrus sinensis* (Miyata *et al.* 2012), *Medicago sativa* (Neda *et al.* 2012), muskmelon (Shripad *et al.* 2010), *Solanum melongena* (Sidhu *et al.* 2014) and in *Tectona grandis* (Sontikun *et al.* 2013). Bombarding the explants twice increased the number of cells giving GUS events and this could be due to the fact that multiple bombardments allow better coverage of the target areas and compensate for misfire from faulty and poorly set rupture disc as was the case in rice (Wang *et al.* 1988). *GUS* is a superior reporter gene for plant transformation studies. It can easily be assayed histoenzymatically to localise GUS activity in plant cells and tissues. *GUS* is stable, and tissue extracts continue to show high level of GUS activity after prolonged storage (Jefferson *et al.* 1987).

The *AtWBC19* gene confers resistance to antibiotic kanamycin which is present as selection marker upstream of the *GUS* reporter gene. Presence of this gene was confirmed using gene specific primers results in the 2.1 kb band size, hence this confirms the integration of *GUS* gene also. Similar confirmatory results were obtained by Shripad *et al.* (2010), Mentewab and Stewart (2005) who used *AtWBC19* gene as selection marker gene in plant transformation experiments. Selectable markers and visible marker reporter genes rarely affect the studied trait of interest, but provide a powerful tool in determining the success of the transformation events.

Conclusion

To summarize, we have standardized an efficient regeneration protocol for an endangered species *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. Micropropagation studies combined with advances in genetic transformation studies in this species for the present data on high plant regeneration frequency, may eventually lead *C. forskohlii* into bioreactor for the production of subunit vaccines.

Acknowledgements

Authors are highly thankful to Department of Biotechnology (DBT-HUB programme), Government of India, New Delhi, for giving financial assistance to this project and we thank Dr. Neal Stewart, University of Tennessee, USA for providing the pABC construct.

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