A simple and efficient genetic transformation protocol for *Eucalyptus camaldulensis* using internodal explants

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Abstract
A simple and efficient genetic transformation protocol was developed for *Eucalyptus camaldulensis*. Selected line of eucalyptus was established under *in vitro* conditions by using nodal cuttings.Assessment of hygromycin sensitivity on explants collected from *in vitro* established cultures showed that hygromycin concentration of 2.5 mg/L was optimum for transformation. Transformation procedure was standardized with internodal explants through indirect organogenesis. Transformation efficiency of intermodal explants was assessed by plasmids pCAMBIA1305.1 and pRNAiLIC-Hyg carrying GA2ox gene. The transformation efficiency of 20 % was achieved with the present protocol and regeneration of transgenic shoot was obtained in two months of culture compared to three to four months in previous studies.Confirmation of gene integration in eucalyptus was confirmed by histochemical staining and PCR based methods.

Key words: Agrobacterium, Biotechnology, Eucalyptus, *in vitro* regeneration, Transformation.

Introduction

Genetic engineering in trees is considered to be a powerful tool for enhancing traits in woody plants. Genetic transformation in eucalyptus could improve the traits beyond the capability of conventional breeding and surpass the barriers encountered in eucalyptus breeding programs (Dibax et al., 2010). To date, various methods of transformation have been developed in eucalyptus which include *Agrobacterium*-mediated transformation, biolistic gene bombardment, sonication and combinations of these methods with various explants and varying transformation efficiencies. However, so far most of the explants used for eucalyptus transformation were juvenile tissues derived from young seedlings such as seedling segments, cotyledons, hypocotyls or young leaves (Girijashankar, 2011). Although these techniques had yielded large number of transgenics, they were unsuitable for elite or selected hybrid lines. This is because seedlings of selected lines or hybrids would be from a segregating population and transgenics generated from seedlings would result in segregated phenotypes (Rezende et al., 2014; Thanananta et al., 2018).

Eucalyptus camaldulensis is the commonly planted species of eucalypt in arid and semi-arid regions and the second most important species in terms of current annual increment of wood (Eldridge et al., 1993). Commercial applications of genetic engineering in eucalypts would require the development of reliable gene transfer systems. Mullins et al.(1997) had observed a low transformation and regeneration capacity fromthe clonal explants of *E. camaldulensis* and hence introduction of useful genes into this woody species has been delayed in comparison with other plants. Thus, it is important to develop a genetic transformation method for *E. camaldulensis* by

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using clonal explants with improved transformation efficiency.

Material and Methods

Establishment of in vitro cultures and explants preparation

Clone of *E. camaldulensis* (ITC 316)was collected from the nursery of ITC collections maintained in Bhadrachalam, India. The clone was established in pots containing sandand soil mix (1:1) and were maintained in glass house conditions. Nodal explants collected from the three-month old plant were surface sterilized using 4% (v/v) sodium hypochlorite for 10 min, washed three times with sterilized deionized water (dH_2O) and *invitro* established in Murashige and Skoog medium (MS medium) with 2.0 mg/L 6-Benzylaminopurine (BAP) and 3% sucrose. Sub culturing was done with an interval of three weeks and subcultures were incubated at 24°C with 16h light and 8h dark. Initiation of axillary buds started after three weeks. After seven weeks, shoots were formed which was kept for multiplication on MS medium with 0.5 mg/L BAP. At the end of the third month, shoots were transferred on to elongation medium (MS medium with 0.04 mg/L BAP) for another month to obtain elongated shoots. Long shoots were selected from *in vitro* grown cultures after four months of culture establishment and intermodal cutting of 1.0 cm was taken for genetic transformation experiments (Fig. 1).

Hygromycin sensitivity on intermodal explants

Shoots were collected from four month old cultures grown under *invitro* conditions. The internodes were immediately subcultured into basal MS medium containing different concentrations of hygromycin (0, 2.5, 5.0 7.5 and 10.0 mg/L) and maintained for 8 weeks with two-week subculture interval (Table 1). Fifteen segments were used for each treatment with three shoots per bottle and data on callusing and regeneration were recorded from each explant after eight weeks.

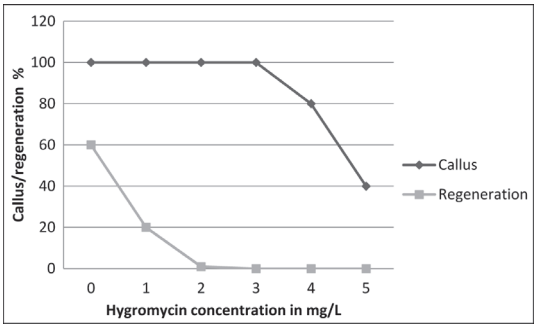

Figure 1. Hygromycin sensitivity on callusing and regeneration from intermodal explants from *E. camaldulensis*. Each data point is the average of 15 explants

Vector constructions

Two vectors pCAMBIA 1305.1 and pRNAiLIC-Hyg-GA2ox were used in this study. pCAMBIA 1305.1 is a binary vector which contains hygromycin resistance and GUSPlus genes within the left and right border sequences in the vector. pRNAiLIC-Hyg-GA2ox was made as follows: A 220 bp region of Ec GA2ox was amplified from *E. camaldulensis* genomic DNA with primers suggested by Dayan et al. (2010). The PCR product was sequenced and found to be derived from the coding region of GA2ox. PCR product was re-amplified with gene specific adaptor primers and directly cloned in to pRNAiLIC vector by the

Table 1. Media used in genetic transformation of *E. camaldulensis*

No.	Name of medium	Composition
1	Basal medium (BM)	MS medium+ 3% sucrose+0.1% caseinhydrolase
2	Pre-incubation medium	BM+10 mg/L zeatin+1.0mg/L NAA+ 100µM Acetosyringone) pH 5.8
3	Co-cultivation medium	BM+10 mg/L zeatin+1.0mg/L NAA+ 100µM Acetosyringone) pH 5.8
4	Selection medium I	BM+10 mg/L zeatin+1.0mg/L NAA+ 250 mg/L Cefotoxime+ 1.0mg/L Hygromcycin
5	Selection medium II	BM+10 mg/L zeatin+1.0mg/L NAA+ 250 mg/L Cefotoxime+ 2.5 mg/L Hygromcycin
6	Multiplication medium	MS+0.25 mg/L BAP+ 250 mg/L Cefotoxime+ 2.5 mg/L Hygromcycin
7	Elongation medium	MS+5 mg/L NAA + 250 mg/L Cefotoxime+ 2.5 mg/L Hygromcycin
8	Rooting medium	0.5X MS + 1.0mg/L IBA+ 250 mg/L Cefotoxime

procedure suggested by Xuet al. (2010). The resultant vector was digested with XhoI to release *nptII* gene. Hygromycinphosphotransferase *(hpt*) gene was excised from pCAMBIA1305.1 by XhoI digestion. Digested vector and hpt gene was ligated (ligase1 U/reaction) to derive pRNAiLIC-Hyg-GA2ox.The vector was mobilized to agrobacterium strain AGL-1 by freeze thaw method (Holsters et al., 1978).

Agrobacterium mediated genetic transformationand hardening conditions

Explants were cultured for two days in pre-incubation medium(Table 1) before the transformation process. *Agrobacterium* strain AGL-1 harboring either pCAMBIA 1305.1 or pRNAiLIC:GA2ox was cultured in an LB broth supplemented with rifampicin (10 mg/L) and kanamycin (50 mg/L) under 150 rpm shaking at 28^0C for 20-24 h. The cells were harvested by centrifugation at 4000 rpm for 10 min andre-suspended in liquid MS medium pH 5.0 (OD600 = 0.6).Internodal segments were placed in the *Agrobacterium* cell suspension for 15 min at 50 rpm shaking conditions. The segments were briefly drained on sterilized filter papers to remove excess *Agrobacterium* cells, placed onto co-cultivation medium and incubated under dark condition for 2 days. Co-cultivated segments were then treated with an antibiotic solution (250 mg/L cefotaxime in sterilized dH_2O) for 15 min to eliminate Agrobacterium, and excess water was drained using sterilized filter papers. The segments were then transferred onto solid selection medium I containing hygromycin (1.0mg/L) for one week. Explants were subcultured on to selection medium II (hygromycin 2.5 mg/L) for two rounds at three week intervals. The putative transgenic shoots emerged from the explants were excised out and shifted to the multiplication medium for another month. Shoots were subculturedon to elongation medium for one month followed by subculturing on rooting medium (Table 1) for root initiation. All subcultures were done with an interval of 21-25 days and incubated at 24°C with 16h light and 8h dark.The transformation rate was calculated as a percentage of transgenic shoots or plantlets per explant as suggested by Matsunaga et al. (2012). The various media used in genetic transformation and their composition are listed in Table 1.

Rooted plants were transferred to paper cups containing coco peat and were kept in special structures covered with polythene sheet so as to ensure high humidity. The structures were maintained in glass house with 80% shade. Water was sprinkled inside the polythene structure regularly to maintain the humidity. After one month plants were shifted to a medium containing sand: red soil: FYM in 1:1:1 ratio.Glass house used for hardening was of BSII standard, and IBSC and GEAC approval was obtained for the experiment. The transformed plants were maintained in the green house by regular pruning and shoots and leaves removed were incinerated as per biosafety guidelines.

Histochemical assay and molecular analysis

Shoots which were hygromycin resistant and PCR positive for *uidA* gene(Saha et al., 2012) were subjected to GUS staining protocol as suggested by Jefferson et al. (1987). In case of transgenic GA2 ox lines, presence of the transgene was verified by PCR using *EcGA2ox* gene specific primers on genomic DNA isolated from shoots of transgenic plantlets.

Results and discussion

Eucalyptus is one of the most widely planted hardwood trees in the world because of its superior growth, adaptability and wood properties. Conventional methods in eucalyptus improvement is limited by their long breeding cycles and high levels of heterozygosity (Teulieres et al.,1994; Mac Rae and vanstaden, 1999). Genetic modification of eucalyptus for pulp and wood characteristics has great impact on improving the productivity of plantations (Unnikrishnan and Gurumurthy, 2015).

Establishment of in vitro cultures and hygromycin sensitivity of explants

Juvenile nodal explants from *E. camaldulensis* were used for initiation of cultures on MS medium with 2.0 mg/L 6-Benzylaminopurine (BAP). Initiation of auxillary buds started after three weeks. After seven weeks, shoots were formed which was kept for multiplication on MS medium with 0.5 mg/L BAP. At the end of the third month, 6-8 shootswere observed from each explant. Shoots were transferred on to elongation medium (MS medium with 0.04 mg/L BAP) for another month to obtain elongated shoots. We could establish *in vitro* cultures within four months of initiation of cultures. The explants were collected by taking the internodal region from the shoots and minimum of three explants were obtained from each shoot (Fig. 1).

Girijashankar (2012) reported shoot induction cum multiplication media (SIM) for selected lines of *Eucalyptus camaldulensis*. SIM media was composed of Murashige and Skoog (MS) basal medium supplemented with 2 mg/L benzyl aminopurine (BAP) and 0.1 mg/L naphthalene acetic acid (NAA) and best shoot elongation response was observed on half strength MS fortified with 0.5 mg/L BAP. Fernando et al. (2016) developed *in vitro* cultures from three field-grown adult clones of *E. polybractea* and it was found that over 95% of the resultant leaf explants initiated callus in a medium supplemented with thidiazuron and 1-naphthaleneacetic acid. Ninety percent of callus from all three clones regenerated shoots in a medium supplemented with 6-benzylaminopurine The sensitivity of internodes againsthygromycin was determined by culturing the internodal explants on MS medium supplemented with varying concentration of hygromycin(Fig. 2). There was callus formation throughout the explant when explant was cultured on medium with no hygromycin and 60% of explants had regenerated on the same medium (10-15 regenerants/explant). At 1.0mg/L, though callusing was observed, the number of regenerants was reduced drastically (two to three/ explant). At concentration of 2 mg/L,

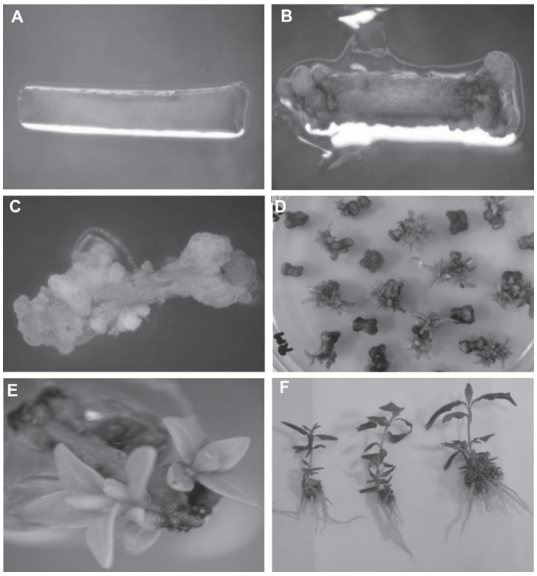

Figure 2. Regeneration of transgenic Eucalyptus from internodal explant**.** A. Internodal explant B-Initiation of callus (15 days after culture) C- Proliferation of callus (30 days after culture) D-Shoot regeneration (60 days) E-Fully grown shoot F-Rooted plants

regeneration was completely arrested with profuse callusing. As the regeneration was not observed at 2.0 mg/L concentration and proliferation of callus started diminishing after 3 mg/L, concentration of 2.5 mg/L was used for selection of transformed shoots in the study.

Hygromycin is known to be more toxic to plant tissues even at low concentration compared to kanamycin and chances of selection escape is low in hygromycin compared with kanamycin. There are only limited reports on eucalyptus regeneration using hygromycin as selection antibiotic (Ma et al., 2011; Fernando et al., 2016). Deepika et al. (2011) obtained efficient callus induction and regeneration of shoots in elite clonal lines of eucalyptus clonesusing hygromycin selection. Fernando et al (2016) reported efficient regeneration protocol for adult derived explants of *E.polybractea* using *hpt*gene. However, there was no study in case of *E. camaldulensis* using hpt gene as resistant gene used for transformation. Different hygromycin

concentration was tried initially to arrive at optimum concentration of hygromycin to be used in transformation. Depending on the genotype used there could be variation in induction of callusing and regeneration capacity and hence it was essential to check optimum concentration by using the range used in previous studies (Deepika et al., 2011).

Standardization of transformation protocol

Proliferation of callus started after two weeks of culture on selection medium II. The callus was compact and possessed nodular structures (Fig 2B). Initially, calli were of pale yellowish colour, and developed patches of reddish colour over time. Regeneration from callus was observed at the end of one month of culture. Multiple shoots were regenerated throughout the internodal segment and were subcultured in to multiplication medium.The control explants were healthy and green on regeneration media without any selection. However, they turned necrotic once transferred toselection medium after 5 days. The various stages of callusing and regeneration of *E. camaldulensis* after transformation are depicted in Fig. 2. We had used concentration of 1.0 mg/L as hygromycin concentration in selection medium I. This had resulted in better callusing than 2.5 mg/L. Hygromycin concentration of 2.5 mg/L was continued in rest of the medium used in thetransformation process except for the rooting medium. However, we made stringent selection at the shoot regeneration stage for two rounds with hygromycin concentration of 2.5 mg/L and thus ensuried the possibility of taking forward only transformed tissues.

Elongation and rooting of transgenic shoots

Transgenic shoot was excised from the explant and was transferred to elongation medium with 5 mg/L NAA. Shoots were maintained in elongation medium for a month and subsequently shifted to rooting medium for 2-3 weeks. Different media compositions were used among which the best response for rooting was observed in half MS basal supplemented with 1.0mg/L IBA (Table 1). Concentration of IBA of more than 1.0mg/L resulted in callus formation. The number of roots varied from 2 to 6 per shoot and shoots obtained from long term held cultures responded immediately. It was found that a well-formed shoot of 6.0 cm length with a pair of roots (4.0 cm) length could withstand hardening conditions well.

Confirmation of transformation and efficiency

Two constructs pCAMBIA 1305.1 and pRNAiLIC-Hyg-*EcGA2ox* were used to check the efficiency of present transformation protocol. These were two different vector backgrounds and hence it was required to check the efficiency of process under two different vectors. Putative transgenic calli from explants transformed with pCAMBIA 1305.1 were subjected to histochemical staining. Multiple calli were stained as blue indicating expression of â-glucuronidase gene (*uidA*/gusA) (Fig. 3A). GUSPlus gene inpCAMBIA 1305.1 is a *gus*gene interrupted by *cat 1* intron of 200 bases to make its expression limited to eukaryotic cells only. Hence, it could be assumed that *uidA* expression was from transformed plant cells as splicing of intron was necessary before its expression. The presence of

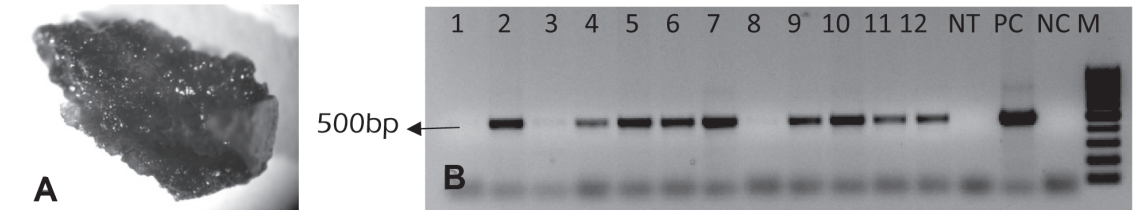

Figure 3. Confirmation of *uid* gene by histochemical staining and PCR verification of transgenic eucalyptus by gene specific primers

A. 30 days old transgenic calli stained with GUS stain B. Gel image on presence of transgene in eucalyptus transformed with pRNAiLIC GA2ox (NT- Non transgenic DNA, PC-Positive control, NC- Negative control)

Table 2. Genetic transformation in *E. camaldulensis* with two plasmids *viz*., pCAMBIA1305.1 and pRNAiLIC-GA2 ox

	pCAMBIA1305.1	pRNAiLIC-GA2ox
Total internodal explants	50	100
Hygromycin resistant shoots	16	35
Shoots with PCR positive for gene	11	21
Percent transformation for the gene	22	21

hygromycin gene in selected line was confirmed by amplification of *Hyg* gene by gene specific primers. There were sixteen hygromycin resistant lines on transformation with pCAMBIA 1305.1 from 50 internodal explants. Eleven out of 50 explants tested positive for the *uidA* gene making a transformation efficiency of 22% (Table 2). DNA from non-transgenic plant did not show any amplification of *uidA* gene. In case of pRNAiLIC-Hyg-GA2ox construct, a transformation efficiency of 21% was obtained. We could get 21 shoots positive for GA2ox transgene out of 100 internodal explants.The presence of transgene in plants transformed with pRNAiLIC-Hyg-GA2ox was confirmed by PCR. All transgenic lines showed the presence of expected amplicon (500 bp) as depicted in Fig.3B.

The increased transformation rate in the present method could be attributed to various factors such as preculture conditions, type of antibiotic and nature of explant. Choosing explant is one of the key considerations for obtaining high transformation frequency. Previously, *Eucalyptus* was transformed mostly by using juvenile tissues obtained from young seedlings and the efficiency of transformation was generally less than 10% in addition to lack of application to selected lines because of explants derived from young seedlings. A maximum transformation efficiency of 7 and 10 % were reported for *E. globulus* and *E. camaldulensis* using hypocotyls as explants (Ho et al., 1998; Matsunaga et al., 2012) while it was only 2 and 6 % efficiency for *E. terticornis* and *E. camaldulensis* when leaf was used as explant (Mullins et al., 1997; Aggarwal et al., 2010). The transformation frequency could be enhanced by preculturing of explants as reported in *E. terticornis* (Prakash and Gurumurthy, 2009).Thanananta et al. (2018) reported an efficiency of 24 per cent from high proliferating nodal explants of *E. camladulensis* x *E. terticornis* FI hybrid lines. However, occurrence of chimeras was quite high when proliferating tissues were used for genetic transformation. Here, we used internodal stem segments as explants and hence chances of chimeras was quite low compared to nodal explants.

Hardening of transformants

Rooted plants were transferred to paper cups containing coco peat and maintained in special structures with polythene sheet for a month. During the first two weeks of hardening, defoliation of *in vitro* formed leaves was observed which was followed by root elongation along with induction of new leaves. Root growth was more prominent than shoot growth under hardening conditions. Humidity in chamber was gradually decreased by making holes in the polythene structures and removing the shade gradually. The process was continued for two weeks and healthy plants were shifted to medium containing sand: red soil: FYM in 1:1:1 ratio. Similar results were obtained by Girijashankar (2011) in *E. camalduelnsis* during hardening of *in vitro* established non transformed plants.

We could establish elongated shoots for collection of intermodal explants under *in vitro* conditions. Minimum of three internodes were obtained from one elongated shoot and there could be three or four shoots per tissue culture container, thus ensuring enough explants for commercial production. Additionally, the present method could be usefulfor genetic transformation of any promising line or hybrid line. Usually methods that require callus formation and organogenesis require 90-120 days to obtain transgenic shoots as reported by previous

workers (Mullins et al., 1997; Dibax et al., 2010; Matsunaga et al., 2012).Our method requiredonly 60 days to obtain transgenic shoots from intermodal explant with efficiency of more than 20%, making it a simple and efficient protocol for transformation of *E. camaldulensis*.

A simple and efficient transformation protocol was developed for genetic transformation of *E.camaldulensis* and efficiency of the process was evaluated by transformationwith two plasmids.Two different plasmids gave comparable results in terms of efficiency of the protocol used. As the method did not require juvenile tissues, the method could be used for transformation of any promising line. The method could benefit genetic improvement of *E. camaldulensis* for the selected traits of interest.

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