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# Exogenous gene transfer in Assam tea [*Camellia assamica* (Masters)] by agrobacterium-mediated transformation using somatic embryo

**Hijam Ranjit Singh[1*], Neelakshi Bhattacharyya[1], Niraj Agarwala[1], Prasenjit Bhagawati[1], Manab Deka[2] and Sudripta Das[3]**

[1]*Department of Biotechnology, Tocklai Tea Research Institute, Jorhat, Assam, India*
[2]*Department of Biological Sciences, Gauhati University, Assam, India*
[3]*Institute of Bioresources and Sustainable Development, Takyelpat, Manipur, India*

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**ABSTRACT**

*A transformation protocol has been standardized in tea (Camellia assamica (Masters), the most economically important perennial woody plant in India, with Agrobacterium tumefaciens strain, LBA 4404 (pCAMBIA 2301; pCAMBIA 1301). This method could be exploited for expression of exogenous gene in tea plants in 15-18 month period. Development of secondary somatic embryos were observed after agroinfection and tested positive for neomycin phosphotransferase II (nptII) and glucuronidase (gusA) genes, regenerated on regeneration media and subsequently multiplied the regenerants. The transformation frequency of A. tumefaciens (LBA 4404) with the binary vector pCAMBIA 2301 and pCAMBIA 1301 on the basis of antibiotic resistance was found to be 2.5% and 3.3% respectively. Further analysis of antibiotic resistant plantlets was done by GUS histochemical assay and polymerase chain reaction (PCR). The PCR positive putatively transformed plantlets were confirmed by Southern blotting for exogenous gene transfer.*

**Key words**: Agrobacterium, transformation, *Camellia assamica*, tea, transformation frequency.

**ABBREVIATIONS:** ***ddw****, double distilled water;* ***IM****, induction medium;* ***MS****, Murashige & Skoog;* ***SEM****, somatic embryogenesis medium;* ***BAP****, benzyl amino purine;* ***NAA****, napthyl acetic acid;* ***d****, days;* ***CM****, coculture medium;* ***SE****, somatic embryo;* ***SSE****, secondary somatic embryo;* ***RM****, regeneration medium;* ***IBA****, indole butyric acid;* ***GA3****, gibberellic acid;* ***MM****, multiplication media;* ***CTAB****, cetyl trimethylammonium bromide;* ***GUS****, glucuronidase;* ***gusA, GUS exonA****;* ***nptII,*** *neomycin phosphotransferase gene*

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## INTRODUCTION

Tea is a perennial woody plant; the tender leaves are used to make black or green tea. Tea, which belongs to the family Theaceae, is one of the most important woody plantation crops yielding a non-alcoholic beverage. According to Wight's nomenclature [1,2], tea can be classified into three races: 1) *Camellia sinensis* L. or the China tea plant, 2) *Camellia assamica* (Masters) or the Assam tea plant and 3) *Camellia assamica* sub sp. lasiocalyx (Planch. MS) or the *Cambodiensis* or Sounthern form of tea plant. Due to the presence of high levels of polyphenols, tea exhibit low competence for transformation as well as regeneration [3]. Foreign genes have been introduced into cells of several woody crops by using the *Agrobacterium tumefaciens* Ti plasmid [4]. Genetic transformation is a way to develop plants with lengthy generation and breeding cycles, such as tea. Conservative breeding is limited in tea because of

the long gestation period and cross-pollination [5]. Biotic and abiotic stress-resistant mutants are not obtainable in tea, making conventional breeding inadequate and time consuming for crop improvement [6]. Of the existing genetic transformation methods, *Agrobacterium*-mediated gene transfer is the most advantageous because it produces defined incorporation of transgenes, low copy number, and preferential integration of transcriptionally vigorous chromosomal regions [7].

The natural ability of the phytopathogenic *Agrobacterium* for gene transfer to plants has been exploited recently for the genetic engineering of several woody tree species, including rubber [8], "Royal Gala" apple [9], kiwifruit [10] and almond [11]. A successful molecular breeding approach through *Agrobacterium*-mediated genetic transformation would represent a significant step in overcoming the existing constraints in tea improvement programmes. Thus, there is a need to develop an efficient, reproducible, and relatively simple genetic transformation protocol in tea. Taking reporter genes transformation on tea (*Camellia sinensis* (L) O. Kuntze) has been done by many workers [6, 12, 13, 14] but there is hardly any report about *Agrobacterium*-mediated transformation taking *Camellia assamica* (Masters). With this objective, the authors have developed a rapid, efficient, and quite economical *Agrobacterium*-mediated infiltration method of somatic embryo transformation in tea. The feasibility of the method has been documented through introduction of *gusA* and *nptII* genes.

The use of reporter gene simplifies the expression of the gene in transgenic plants and is widely used as a scorable marker. Normally, the glucuronidase (GUS) gene is absent in plant tissues and no detectable background was obtained in most of the higher plant cells. The sensitivity of the assay makes this marker useful for verification of transformation [15].

## MATERIALS AND METHODS

### 2.1 Plant Materials

The germplasm selected for the present study was Assam type tea *Camellia assamica* (Masters) Tocklai Vegetative clone, TV21, the only quality clone, [16] collected from the New Botanical Area at Tocklai Tea Research Institute, Jorhat (Assam), India.

### 2.2 Production of Tea Somatic Embryos

Developing and mature seeds were collected, removed the fruit coats and dipped in water for overnight as described by Mondal *et al*., 2001 [6] and Lopez *et al*., 2004 [12]. Washed the cotyledons with Tween-20 for 5 min and then rinsed with ddw for 2–3 times. Washed cotyledons were treated with 0.3% Blitox (Rallis India) by shaking the container for half an hour and then rinsed with ddw for 5–6 times. Thereafter, washed cotyledons were surface-sterilized with 0.1% aqueous mercuric chloride for 10 min under sterile conditions and then washed with sterile double distilled water, dried on sterile filter paper and used as explant for somatic embryo development. Surface-sterilized cotyledons were then cultured on IM containing full-strength basal MS salts [17], 20 $g.L^{-1}$ sucrose, and 0.65 % (w/v) agar (pH 5.6-5.8). After a month, somatic embryogenic calli derived from the cotyledons were transferred to SEM) containing half-strength basal MS salts, 20 $g.L^{-1}$ sucrose, 0.65 % (w/v) agar, and 1 $mg.L^{-1}$ BAP and 2 $mg.L^{-1}$ NAA with pH 5.6-5.8. The secondary somatic embryos developed thereafter were maintained in culture laboratory conditions of 25 ± 2°C and 16 h photoperiod (70 µmol $m^{-2}$ $s^{-1}$) with light provided by cool fluorescent tubes at 1500 lux and 55 ± 5% relative humidity. The SEs were regularly subcultured after every 30 days for their maintenance. The globular SEs having high potential for repetitive embryogenesis and conversion were used as explants for subsequent *Agrobacterium* infection.

### 2.3 Bacterial Strains and Plasmid

*Agrobacterium* strain, LBA 4404 (Invitrogen) and pCAMBIA 2301 and pCAMBIA 1301 (http://www.cambia.org.au/) with the β-glucuronidase (GUS) gene interrupted with catalase intron driven by the Cauliflower Mosaic Virus 35S (CaMV 35S) and nopaline synthase terminator as selectable marker gene was used for the transformation. Binary vector pCAMBIA 2301 has neomycin phosphotransferase II (*npt*II) selectable marker under the control of CaMV 35S promoter and *nos* terminator in T-DNA region (Figure 1 & 2) which confers resistance to the antibiotic kanamycin as a plant selection marker. Kanamycin resistance gene located outside the T-DNA region confers resistance to the antibiotic kanamycin as a bacterial selection marker. On the other hand, pCAMBIA 1301 has hygromycin resistance gene (*hyg*R) for plant selection and kanamycin resistance gene located outside the T-DNA region for bacterial selection (Figure 3 & 4). The plasmids, pCAMBIA 2301 and pCAMBIA 2301 were individually mobilized into electrocompetent *A. tumefaciens* strain, LBA4404 by electroporation.

Validation of the plasmid was done by colony PCR of recombinant *A. tumefaciens* strains with *gus*A and *npt*II gene specific primers, restriction digestion of plasmids isolated from the bacterial strains and resolved in 1% agarose gel stained with ethidium bromide (0.5 µg.mL$^{-1}$).

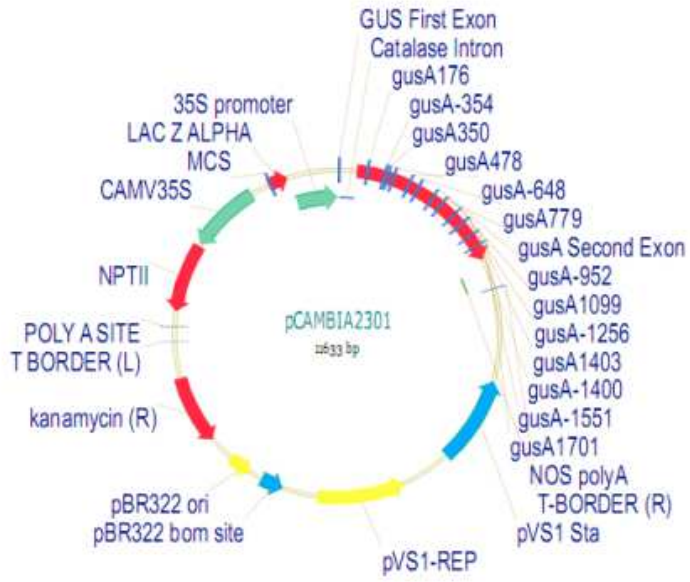


**Figure 1: Schematic diagram of the plasmid pCAMBIA 2301. The binary vector pCAMBIA 2301 (CSIRO, Australia) harboring the reporter *gus*A and *npt*II genes driven by the CaMV 35S promoter**

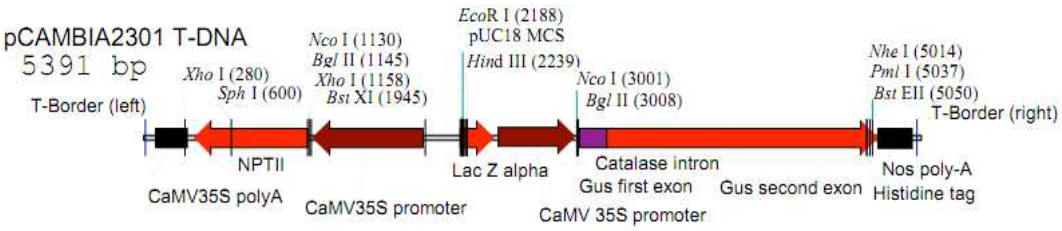


**Figure 2: T-DNA region of pCAMBIA 2301**

### 2.4 Tea Transformation, Selection and Regeneration

A fresh culture of *Agrobacterium* was obtained by inoculating a single colony in 50 mL TY medium (0.5% tryptone, 0.3% yeast extract, pH 7.0) containing 50 mg.L$^{-1}$ kanamycin and grown overnight in the dark at 28°C at 180 rpm. Bacterial OD 0.6 and cell density ($10^9$.ml$^{-1}$) was maintained for maximum transformation efficiency [6]. The bacterial suspension was pelleted by means of centrifugation (2000g, 10 min) and re-suspended in IM containing MS basal salts and 20 g.L$^{-1}$ sucrose. SEs were cut into small pieces (0.5 cm × 0.5 cm) and immersed in the bacterial suspension for 20 min in a rotary shaker. Then the explants were blotted dry on sterile blotting paper to remove excess bacterial suspension. Explants were cultured on liquid CM containing half-strength basal MS salts and 20 g.L$^{-1}$ sucrose with 400 µM.L$^{-1}$ acetosyringone for 2-6 d in the dark at 25±2°C.

After co-cultivation, explants were washed with ddw containing cefotaxime (1000 mg.L$^{-1}$), dried on filter paper and transferred onto SEM supplemented with 25, 35, & 50 mg.L$^{-1}$ kanamycin, 15, 25 & 35 mg.L$^{-1}$ hygromycin and 400, 200, 0 mg.L$^{-1}$ cefotaxime for three selection cycles depending upon the type of binary vector used. For antibiotic selection the SSEs were regularly subcultured to fresh SEM medium in two-week intervals with gradually decreasing in cefotaxime concentration and gradually increasing in kanamycin and hygromycin concentration. Antibiotic resistance SSEs were then shifted to RM containing half-strength basal MS salts, 20 g.L$^{-1}$ sucrose, 0.65 % (w/v) agar, and 6 mg.L$^{-1}$ BAP, 0.5 mg.L$^{-1}$ IBA and 2 mg.L$^{-1}$ GA3 with pH 5.6-5.8. After regeneration of secondary embryos they were transfer to MM containing half-strength basal MS salts, 20 g.L$^{-1}$ sucrose, 0.65 % (w/v) agar, 5

mg.L$^{-1}$ BAP , 0.5 mg.L$^{-1}$ IBA and 1 mg.L$^{-1}$ GA3 with pH 5.6-5.8 and supplemented with the same increasing in the concentration of kanamycin and hygromycin as employed during embryogenesis for three different selection cycles. Forty five days old SSEs and nine months old putatively transformed plants were used for GUS assay and molecular analyses analysis respectively.

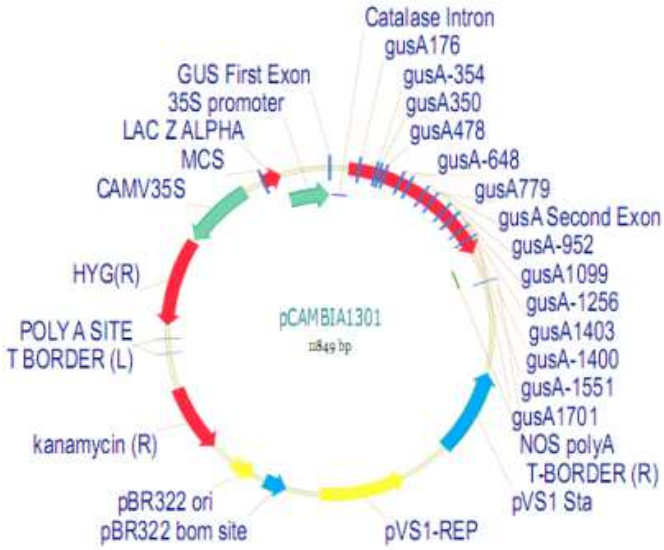


**Figure 3: Schematic diagram of the plasmid pCAMBIA 1301. The binary vector pCAMBIA 1301 (CSIRO, Australia) harboring the reporter *gus*A and *hyg*(R) genes driven by the CaMV 35S promoter**

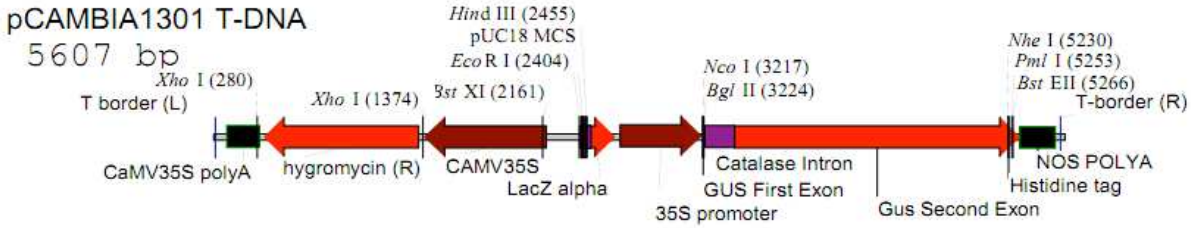


**Figure 4: T-DNA region of pCAMBIA 1301**

**2.5 GUS Histochemical Assay**

The transgene gene expression analysis was checked by histochemical assay [15] with the help of β-Glucuronidase Reporter Gene Staining Kit (Sigma) according to manufacturer's instruction. The analysis was done both for putative transformants and untransformed control plantlets.

**2.6 PCR Analysis**

Total genomic DNA was isolated from putatively transformed and untransformed leaves using CTAB method [18]. Primer set used for amplification of 148 bp fragment of partial sequence of *npt*II gene was nptIIF: TAGCCGGATCAAGCGTATG and nptII R: CTGTGCTCGACGTTGTCACT for checking kanamycin (LBA 4404 WITH pCAMBIA 2301) plantlets; the primers used for amplification of 183 bp of partial sequence of *gus*A gene were gusF: GTTACAAGAAAGCCGGGCAA and gusR: ACCCACACTTTGCCGTAATG for checking hygromycin (LBA 4404 WITH pCAMBIA 1301) plantlets. Primer pair (ChvA-F: TCCATCAGCAACGTGTCGGTGCT and ChvA-R: GTGGAAAGGCGGTGAGCGATGAT) designed from the *chv* region of *Agrobacterium* was used to detect the bacterial contamination. PCR was carried out in 25 µl of reaction containing 50 ng of template DNA, 2.5 mM of dNTPs, 1 U of *Taq* DNA polymerase, 1X *Taq* DNA polymerase buffer containing 15mM $MgCl_2$ and 20 pM of gene specific primer set. PCR was performed with initial denaturation of 94° C for 5 min, 34 cycles of 94° C for 30 sec, 54° C for 30 sec for *gus*A (51° C for 30 sec for *npt*II), 72° C for 30 sec and final extension of 72° C for 7 min and electrophoresed on 1.5% agarose gel.

**2.7 Southern Blot Analysis**

For Southern hybridization analysis, total DNA was isolated using the same method as PCR analysis. DNA of PCR-positive transgenic was used to be sample to amplify *gus*/*npt*II fragment. The amplified products were separated on 1.5% agarose gels overnight, and the fragements were transferred onto nylon membranes (Roche) using standard protocols. Southern hybridization probes (specific fragment of *gus*/*npt*II) were labeled with DIG using the DIG DNA Labeling and Detection Kit (Roche), following the manufacturers protocol. Hybridization and immunological detection were carried out according to the instruction of manufacturer (Roche).

## RESULTS

**3.1 Somatic Embryogenesis of Tea**

The establishment of tea somatic embryos and regeneration system was carried out by surface-sterilization of fresh tea cotyledons. This involves inoculation of fresh tea cotyledons in IM (**Figure 5A**). After 20 days, the emergence of embryonal axis was observed and removed, and cotyledons were maintained in IM for the induction of primary SEs (**Figure 5B**). In this way direct somatic embryogenesis was established from cotyledonary explants of different ages and genotypes. These embryos were subcultured in SEM. Within 3 months SEs were directly formed from the epidermal tissues of the initial embryos without callus formation (**Figure 5C**). These somatic embryos were further multiplied and maintained in SEM (**Figure 5D**). Repeatable embryogenesis could be achieved and secondary embryogenesis was obtained predictably and repeatedly in a media combination (IM followed by SEM), which could be applied over a wide range of germplasms.

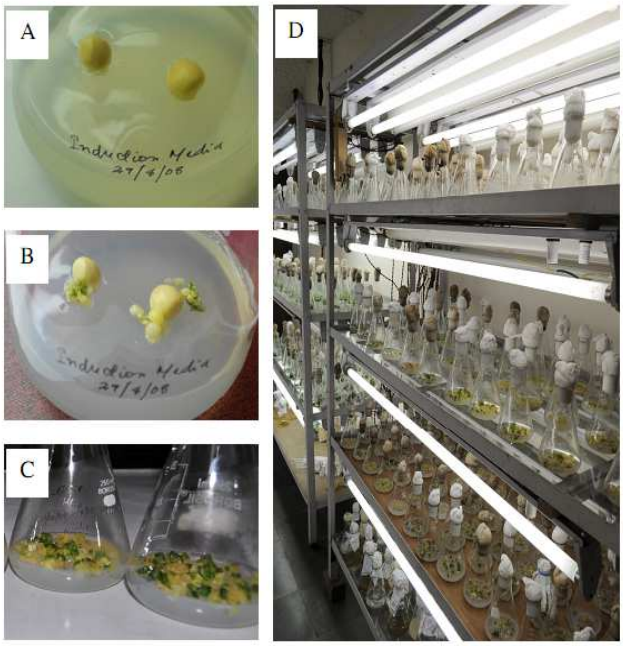


**Figure 5: Somatic embryogenesis of tea. A) Inoculation of cotyledons in IM media. B) and C) Production of SEs from cotyledons in SEM media. D) SEs maintained in tissue culture rack**

**3.2 Studies on pCAMBIA 1301 & pCAMBIA 2301**

**3.2.1 Transformation of plasmid pCAMBIA 1301 & pCAMBIA 2301 to *A. tumefaciens* (LBA 4404)**

Plasmid DNA was mobilized into electrocompetent cells of *Agrobacterium* by electroporation and incubated at 28° C for 2 days; 50 - 60 colonies were selected on TY plates containing 50 µg.ml$^{-1}$ kanamycin. The plasmid was isolated, a single band of 11633 bp and 11849 bp were obtained on restriction digestion with *Bgl*II (**Figure 6**). Colony PCR gave the amplification of 183 bp and 148 bp of partial sequence of *gus* and *npt*II gene respectively (as predicted by Primer3 software) when the amplified products were resolved in 1% (w/v) agarose gel (Figure not given in the text).

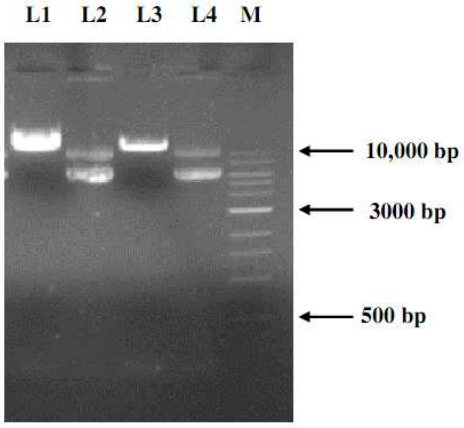


**Figure 6: Restriction digestion of pCAMBIA 2301 and pCAMBIA 1301 isolated from transformed *Agrobacterium* LBA 4404. M: New England Biolabs, 1Kb ladder, L1: pCAMBIA 1301 digested with *bgl*II; L2: undigested pCAMBIA 1301; L3: pCAMBIA 2301 digested with *bgl*II; L4: undigested pCAMBIA 2301**

**Table 1. Restriction digestion mixture**

| Components | Reaction mixture |
|---|---|
| DNA (pCAMBIA 2301/pCAMBIA 1301) | 10µl (3 µg) |
| *Bgl*II(20U.µl$^{-1}$) | 0.3 µl |
| Buffer (10X) | 2 µl |
| Sterile ddw | 7.7 µl |
| Total | 20 µl |

### 3.2.2 Transformation experiments with tea using *Agrobacterium* strain LBA4404 (pCAMBIA 2301) and LBA4404 (pCAMBIA 1301)

Transformation of SEs were carried out at 20 min time interval and transformation frequency was recorded by counting the number of antibiotic (kanamycin, hygromycin and cefotaxime) resistant secondary SEs obtained after selection cycle. Two hundred thirty four and two hundred twenty eight antibiotic resistant SSEs were obtained from 100 explants co-culture after agroinfection with *Agrobacterium* strain LBA4404 (pCAMBIA 2301) and LBA4404 (pCAMBIA 1301) respectively after three cycles of selection (**Table 2**). Within 3 months of culture, the untransformed SEs were turned brown and died (**Figure 8A & B**). However, putative transformants were able to proliferate and survived further on applied selection dose (**Figure 8A & B**). After about 5 months of transformation, tea microshoots were started to emerge from the epidermal surface of each of the putative transformant SEs on SEM. On the same media combination, small tea plantlets were developed from these transformants (**Figure 8B**). The overall transformation efficiency of tea with LBA4404 (pCAMBIA 1301), 3.3% ( calculated by dividing the total number of antibiotic resistant plantlets surviving selection cycle by the total number of SSEs developed) was also more efficient than that with LBA4404 (pCAMBIA 2301), 2.5% as given in **Table 2.**

**Table 2. Comparision of transformation efficiency (after antibiotic selection) of tea at O.D 0.6 and 20 min agroinfection time with LBA 4404 (pCAMBIA 2301) and LBA 4404 (pCAMBIA 1301)[a]**

| Vector | Total no. of explants co-cultivated | Total no. of SSE developed ±SE | Total no. of antibiotic resistant SSE ±SE | Total no. of regenerants after selection stress ±SE | Total no. of antibiotic resistant plantlets ±SE | Transform-ation efficiency (%) |
|---|---|---|---|---|---|---|
| pCAMBIA2301 | 100 | 2223 ±1.15 | 234 ± 1.73 | 122 ±0.57 | 56 ±0.57 | 2.5 |
| pCAMBIA1301 | 100 | 2010 ±0.57 | 228 ±0 | 128 ±1.15 | 67 ±0.57 | 3.3 |

[a] *Values are the mean of three readings; SE, standard error.*

### 3.3 Confirmation of Putative Transformants

### 3.3.1 Histochemical GUS staining

The transgenic nature of SSE was confirmed by means of histochemical localization of the *gus*A gene. Some of the antibiotic resistant SSEs obtained after three cycles of selection were randomly selected to check for the presence of β-glucuronidase enzyme. Blue coloration was observed in case of putative transformants while untransformed ones

were GUS negative (**Figure 7**). GUS staining is an efficient and reliable indicator of plant genetic transformation. The *gus*A gene is not expressed in *Agrobacterium* because it has an intron [19].

A)   B)

**Figure 7: Histochemical GUS assay of transformed (A: LBA 4404 with pCAMBIA 2301; B: LBA 4404 with pCAMBIA 1301) and control plant. Transformed (left tube of A and B), control (right tube of A and B)**

### 3.3.2 Molecular detection of putatively transformed plants

Antibiotic resistant plantlets were randomly chosen for PCR detection. Two of the seven antibiotic resistant plantlets generated target band size of 183 bp with the gusA primers; two out of five antibiotic resistant plantlets generated target band size of 148bp with nptII primers (**Figure 8C & E**). No band was detected in the untransformed control plantlets. The presence of *gus*A and *npt*II genes in putative transgenic plantlet DNA confirmed the validity of the transgenic plantlets. Primer pairs of *Agrobacterium* did not generate any band from the DNA of putative transgenic plantlets (Figure not shown in the text). It confirmed that bacterial contamination was not there. Some of the PCR positive plantlets were randomly selected for Southern hybridization to verify the integration of the transgenes into the genomic DNA. Positive hybridization band was detected in transgenic lines tested (**Figure 8D & F**). No signal was detected in the untransformed control. This result demonstrated that the T-DNA region of the transformed vector was inserted in the genome of transgenic lines.

### 3.4 Genetic Analysis of Transgenic Plantlets

It is established fact that *Agrobacterium*-mediated transformation results in random integration of T-DNA into the host plant cell genome and thus a single transgenic plant can have multiple integration events of T-DNA. Although scoring the number of integration events in transgenic plants by southern hybridization is a routine protocol, it is time consuming and also tedious. Detection using PCR is a simple method for the identification of the transgene. Genomic DNA of putatively transformed plantlets (randomly selected) shown to be positive for GUS were taken and PCR amplified with gusA and nptII specific primers. The amplification of 183 bp and 148bp respectively for partial sequence for *gus*A and *npt*II genes was compared with the positive control (plasmid) and was observed in all GUS positive transformed shoots and no band was observed in the untransformed plants when analyzed in our present study as shown in **Figure 8C-F**. All GUS positive plantlets were also found to be positive for *gus*A and *npt*II.

*Agrobacterium* strains (pCAMBIA 2301 and pCAMBIA 1301 ) in SEM augmented with cefotaxime, kanamycin and hygromycin; B) Different stages of development of SSE in RM supplemented with kanamycin and hygromycin: the initiation of regeneration (yellow arrowed), regenerated plantlet (red arrowed) and the untransformed SSE perished (blue arrowed); C) PCR detection of hygromycin resistant plantlets with *gus* primers (M: New England Biolabs, 100bp ladder, C: Untransformed control, lanes 1 & 2: Putative Transformants); D) Southern hybridization analysis of *gus* PCR-positive hygromycin resistant plantlets; E) PCR detection of kanamycin resistant plantlets with *npt*II primers (M: New England Biolabs, 100bp ladder, C: Untransformed, lanes 4 & 5: Putative Transformants); F) Southern hybridization analysis of *npt*II PCR-positive kanamycin resistant plantlets.

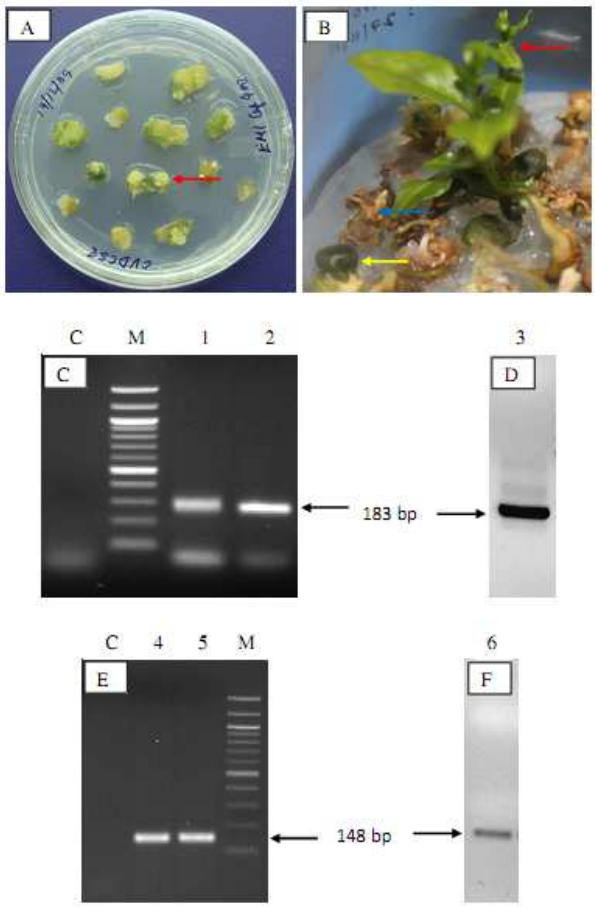


**Figure 8: A) Development of SSE (red arrowed) from SE explant after agroinfection with recombinant**

## DISCUSSION

Transformation and regeneration are major prerequisites for the development of suitable and efficient expression system. Therefore, prior to an experiment, it would be appropriate to have a standardized protocol to maximize the results. Based on the regeneration system, a high efficient *Agrobacterium*-mediated transformation protocol of tea was developed. The ability to form SEs is, in most cases, not merely an intrinsic property of a species. Instead, it is a property under genetic control, such that individual genotypes within a species can differ in their ability to undergo somatic embryogenesis. The composition and concentration of hormones in the culture medium for *in vitro* regeneration of tea have been optimized. Using the regeneration system, SEs could be induced efficiently after somatic embryogenesis of tea cotyledon. And the secondary somatic embryos could persistently give rise to plantlets. The somatic embryos were good starting material for tea transformation. The hormonal combination of 6 $mg.L^{-1}$ BAP), 0.5 $mg.L^{-1}$ IBA and 2 $mg.L^{-1}$ GA3 was found to be best for the regeneration through the matured SE and the absorbance of 0.6 with infection time of 20 min was found to be suitable for tea SE transformation. The transformation frequency reported for the transformation through LBA 4404 (pCAMBIA 2301 and pCAMBIA 1301) on the basis of antibiotic resistance was 2.5% and 3.3% respectively. Stable plant transformation requires a considerable investment in time before the expressed proteins can be analyzed. In contrast, transient gene expression systems are rapid, flexible and straightforward. The transgene expression assays described ensure that most errors and technical problems with gene expression can be identified and resolved before making stable transformants [20]. It was found that none of the control plants gave positive result with X-Gluc indicating the absence of any endogeneous GUS activity, whereas the transformed ones showed the presence of the blue colouration indicative of the expression of exogeneous *gus*A gene. The positive lines were genetically analyzed and integration of the gene into the plant genome was confirmed by PCR [21]. The PCR amplification clearly depicted the desired size of 183 bp and 148 bp band for *gus*A and *npt*II genes respectively and none in the untransformed plantlets. The GUS

positive plants were found to be positive for the presence of transgene, therefore the transformation frequency with *Agrobacterium* LBA 4404 (pCAMBIA 2301 and pCAMBIA 1301) for tea SE was verified and later confirmed by PCR-analysis and Southern blotting analysis. The *npt*II gene was a stable selection marker in plant genetic transformation. However, it was generally agreed that *npt*II was not suitable for the transformation of legumina and monocotyledon plants [22]. Therefore, in order to further eliminate the non-resistant shoots, we prolonged the selection period. The putatively transformed tea plantlets were selected on specific antibiotic containing medium. Some study considered that *npt*II was a suitable selection marker gene for pineapple, but kanamycin was not an appropriate selection antibiotic [22, 24]. They often used hygromycin as selection antibiotic [25, 26]. It was reported that acetosyringone was beneficial for the transformation of monocotyledon [27] which was also found to be same in dicotyledon plant like tea in our present study. There are reports that transgenic plants obtained through organogenesis might be chimeric plants composing of transgenic and non transgenic cells. It is well known that somatic embryogenesis exhibits a virtue of no chimeras, whereas organogenesis shows a merit of little variation [28]. It was reported that the transformed gene caused several side effects at the biochemical level during early stage of plant hardening [39]. So it is meaningful to carefully study the biochemical, physiological, agricultural and ecological characters of transformants in the future.

## CONCLUSION

The transformation and regeneration system described here is simple and effective, allowing routine introduction of gene into *Camellia assamica* (Masters) genome. The efficiency achieved with our protocol may produce hundreds of independent plantlets of transgenic tea within a 15-18 month period. This protocol is followed in our laboratory in order to transform SEs of tea for different objectives like disease resistance transgenic tea, gene knock down of tea transformants, etc.

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