Agrobacterium mediated transfer of nptII and gus genes in Camellia assamica

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The present work is an attempt to develop transgenic hairy root system in Camellia assamica, using leaves infected by Agrobacterium rhizogenes strain LBA9402, harboring binary vector (pART27 and pBI121) carrying β-glucuronidase (gus) as reporter gene and neomycin phosphotransferase (nptII) as selection marker. The transformed hairy roots were grown in phytohormone free MS media in the presence of kanamycin 50 µg/ml. The incorporation and expression of foreign genes were checked by initial gus assay followed by PCR analysis for the presence of nptII and β-glucuronidase (gus) gene. This protocol therefore facilitates the study of gene expression system in general and for root functional genomics in particular, giving scope for transgenic hairy roots as future explants for secondary metabolite production and plantlet regeneration.

Key words: Agrobacterium rhizogenes, transformation, Camellia assamica, nptII, β-glucuronidase (gus).

INTRODUCTION

Tea is a widely consumed non-alcoholic and healthy beverage in the world belonging to the family of Theaceae. Tea is primarily produced from three species of Camellia, Camellia assamica (Assam variety), Camellia sinensis (China variety) and C. assamica ssp. lasiocalyx (Cambod variety) (Balasaravanan et al., 2003). In many countries including India, tea is an important cash crop and has great value as a source of secondary metabolic products. The growing industrialization has always demanded for continual improvement and an increased productivity of this crop but the recalcitrant nature of this woody perennial plant has restricted its scope for genetic improvement unlike other woody perennials, breeding practices followed in tea takes a longer time because of long life cycle and self-incompatible nature of the crop (Banerjee, 1992). Therefore, the effective strategy to overcome the problem could be the use of biotechnological approaches which lessens the constraints for genetic transformation and the time required to obtain a desired character.

The natural responsiveness of plants towards transformation manifested the study for genetic analysis in perennial plant like tea. Although, there are reports mentioning transformation with Agrobacterium tumeficiens and subsequent regeneration in tea by different groups (Joseph et al., 2004). Agrobacterium rhizogenes is a soil bacterium that naturally infects and causes the formation of hairy roots in plant species. These non-malignant tumor-like root systems have been used extensively in genetic transformation studies due to their ease of handling and formation of transgenic plants with high efficiency.

The use of Agrobacterium rhizogenes for transformation has several advantages. It is a non-pathogenic bacterium which can efficiently deliver foreign genes into plant cells without causing any tissue damage. The bacterium is capable of inducing the formation of root masses in various plant species by harboring a binary vector that carries the desired genes. This method allows the introduction of multiple genes into the plant genome, providing a versatile tool for genetic transformation.

The present study aimed to develop a transgenic hairy root system in Camellia assamica using leaves infected with Agrobacterium rhizogenes strain LBA9402. The binary vector used contained the β-glucuronidase (gus) gene as a reporter and the neomycin phosphotransferase (nptII) gene as a selection marker. The transformed hairy roots were grown in phytohormone-free MS media supplemented with kanamycin. The incorporation and expression of foreign genes were confirmed through initial gus assay followed by PCR analysis. This protocol facilitates the study of gene expression in general and provides an opportunity for transgenic hairy roots as future explants for secondary metabolite production and plantlet regeneration.

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Agrobacterium rhizogenes

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rhizogenes mediated hairy root transformation still seems promising for being fast, easy and efficient tool for the study of gene function in economically important crops like tea, where the generation of transgenic plants are difficult and takes a long time. This technique has been instrumental for studying the function of the gene and its expression in plant system in relatively less time.

A. rhizogenes is a gram negative soil bacterium that induces 'hairy root' disease on dicotyledonous plants. Hairy roots are adventitious roots derived from cells transformed by the root inducing Ri plasmid of A. rhizogenes. It is well known for its ability to transfer its T-DNA from the root-inducing (Ri) plasmid to the host genome and thereby inducing hairy root at the site of infection in natural condition. A. rhizogenes transformed roots is characterized by a highly branching root pattern and plagiotropic development (Nilsson and Olsson, 2009). The transformed roots can be grown in vitro as hairy root cultures even after excision from the mother explant. The unrestrained growth, genetic and biosynthetic stability, low doubling time and the ability to synthesize a range of chemical compounds in phytohormone free media makes them a suitable system for in vitro production of secondary metabolites without any seasonal variation. It is a powerful tool for rapid and reproducible research in diverse areas such as, establishment of gene function, analysis of promoter activity, heterologous protein expression, antibody production, modification/overexpression of plant metabolic pathways. Transgenic roots are capable of regeneration into whole viable plants, shunting callus formation and thus avoiding problems of somaclonal variation in a range of plant species (Tepfer, 1990).

Several independent workers have successfully used A. rhizogenes (harboring binary vectors with selection marker) for plant transformations of Lotus corniculatus (Jian et al., 2009), Lithospermum erythrorhizon with a uidA and hptII marker (Yazaki et al., 1998); in Coffea Arabica (Alpizar et al., 2006). Previous studies of A. rhizogenes mediated hairy root formation using leaf as an explant has been successfully reported in tea (John et al., 2009). This has encouraged to explore similar transformation strategies for gene function studies in C. assamica using A. rhizogenes. The present study focuses mainly the use of reporter gene (as visual marker) in tea leaf explants that allows easy selection of transgenic lines through monitoring the function of reporter genes in the transgenic hairy roots developed through infection with A. rhizogenes strain LBA 9402.

**MATERIALS AND METHODS**

**Plant materials**

Young tea shoots from in vitro grown plantlets of C. assamica were taken. The leaves were excised with a sterile blade and wounded at the leaf mid-rib. The aseptic shoot culture are already established from seed cotyledons and maintained on MS medium.

**Bacterial strain and binary vector**

An agropine type, A. rhizogenes strain LBA 9402 and binary vectors pART27 and pBI121 were used for our experiments. The binary vector pBI121 carries nptII gene coding for neomycin phosphotransferase II and a uidA gene coding for β-glucuronidase (gus) driven by cauliflower mosaic virus (CaMV) 35S promoter. The vector pART27 carries the chimeric kanamycin resistance gene along with Tn7 spectinomycin/streptomycin resistance gene for bacterial selection. For transformation A. rhizogenes strain LBA 9402 were made chemical competent by Inoue transformation method (Sambrook et al., 2000) for up-taking the binary vectors pART27 (Gleave, 1992) and pBI121 (Jefferson et al., 1987). For this, 150µl of YMB medium was inoculated with 1ml of an overnight culture of A. rhizogenes and put in a shaking incubator at 125 r.p.m at 28°C until the culture grew to an OD600 of 0.5 to 0.6. The bacterial culture was chilled in ice and centrifuged at 3000 rpm for 10 min at 4°C. The pellet formed was resuspended in ice cold Inoue buffer by gentle swirling and centrifuged at 3000 r.p.m for 10 min at 4°C. The pellet so formed was resuspended (in ice cool Inoue buffer and DMSO) to prepare aliquots and freeze in liquid nitrogen for storage at -80°C. The competent cells were thawed in ice. Plasmid (for each vector) isolated from already transformed DH10B E. coli cells using alkaline lysis method (Sambrook et al., 2000) was used. 1µg of the plasmid was added in separate vials and kept for 10 min incubation at 4°C. This was followed by heat shock at 42°C water bath for 90 s. Fresh YMB medium was added to both the culture and incubated at 28°C for 2 to 3 h with gentle shaking. Both the culture were transferred to YMB agar plates containing kanamycin 50 µg/ml and incubated at 28°C.

**Establishment and selection of hairy root culture**

Single colony from LBA 9402 harboring the binary vector pART27 and pBI121 was inoculated separately in 20 ml of YMB medium containing kanamycin 50 µg/ml at pH 7.0 at 28°C shaking incubator at 100 to 120 r.p.m for 48 h in dark, while YMB medium containing no bacterial inoculation was used as control. Bacterial suspension at exponential growth phase (O.D 0.6) was taken and acetylsyringone (10 mM) was added to the bacterial suspension for about 2 h. This is done mainly to induce the vir genes present in the agrobacterium. The excised leaves were wounded at the leaf mid rib with a sterile blade and kept. Some of the explants were submerged in Petridish along with the bacterial suspension for an hour. The excised infected, leaves were transferred into a new Petridish blotted on a sterile filter paper to remove the excess bacterial suspension, sealed and co-cultivated in dark for about 48 h. The explants were then washed with sterile distilled water for five to six times to eliminate excess bacteria and blotted dry. These infected explants (with their adaxial side down) were then transferred to solidified, phytohormone free MS medium (Murashige and Skoog, 1962) with KNO₃ (380 mg/l), NH₄NO₃ (330 mg/l) and sucrose (30 g/l), at pH 5.7 supplemented with 400 mg/ml cefotaxime (Sigma Chemical Company, USA), to control bacterial overgrowth and kept in dark. The explants were sub cultured after three weeks in phytohormone free MS medium (pH 5.7) (for proliferation) with reduced concentration of cefotaxime (100 µg/ml) and kanamycin (50 µg/ml). The dosage of cefotaxime was reduced gradually from (400, 300, 200, 100, 0 µg/ml) in subsequent sub-cultures and was completely withdrawn after 5th sub-culture.

**Detection using gus assay**

The expression of gus gene in putatively transformed roots was analyzed in root tissues obtained after 65 days of induction according to Jefferson et al. (1987). Roots were washed with sterile
Table 1. Primers taken during the study.

| Primers   | Sequence                        | Annealing temperature |
|-----------|---------------------------------|-----------------------|
| nptII-Forward | TAGCCGGATCAAGCGTATG             | 95°C to 30 s           |
| nptII-Reverse | CTGTGCTCGACGTGTTCACT           | 57°C to 30 s           |
| gus-Forward    | TGTGGAGTATTGCCGCGAA            | 53°C to 30 s           |
| gus-Reverse    | GAGCGTCGCGCAACATTACA         | 72°C to 1 min          |

Figure 1. Effect of hairy root growth at different concentrations of kanamycin.

distilled water and tested for gus expression in X-gluc solution (2 mM X-gluc, 500 mM phosphate buffer, 10 mM EDTA and Triton x-100) for 12 h at 37°C incubation. The explants were soaked in 70% ethanol and examined for detection of blue colour development.

Molecular analysis of transformants

From 5 putatively transformed root clones genomic DNA from about 50 mg tissue was extracted following the method of Doyle and Doyle (1990). The incorporation of nptII and gus genes from the T-DNA of binary vector pART27 (Gleave, 1992) and pBI121 (Jefferson et al., 1987) into tea genome was examined by PCR of gus Staining positive and kanamycin selected root genomic DNA using gene specific primer pairs. Primer designing was done using freely available primer3 software (http://frodo.wi.mit.edu/primer3/) and primers, nptII-Forward-TAGCCGGATCAAGCGTATG, nptII-Reverse CTGTGCTCGACGTGTTCACT and gus Forward TGTGGAGTATTGCCGCGAA, gus-Reverse- GAGCGTCGCGCAACATTACA were taken for analysis. The primers were standardized in Gradient Mastercycler (Eppendorf, Manheim, Germany) and the PCR reaction mixture (20 µl) included 10 pmol forward and reverse primer, 0.5 µl of plant genomic DNA as template, 2 µl 10X Taq DNA polymerase buffer, 0.25 mmol/l dNTPs, 1 U Taq DNA polymerase (Bioline, London, UK). The reaction condition for nptII was 95°C to 30 s, 57°C to 30 s, 72°C to 1 min and for gus was 95°C to 30 s, 53°C to 30 s, 72°C to 1 min for 35 cycles, respectively (Table 1). After amplification the PCR products were separated in an ethidium bromide stained 1% agarose gel and compared with a DNA ladder (New England BioLabs, Beverly, Mass).

RESULTS

Transformation and selection of A. rhizogenes harboring the binary vector in tea

In our study 4 different concentrations (10, 30, 50, and 100 µg/ml) of kanamycin were taken to determine the appropriate concentration for plant selection (Figure 1). The frequency of hairy root infection was higher in kanamycin free media compared to media containing kanamycin 50 µg/ml. Therefore for selection of the explants 50 µg/ml of kanamycin was used which was also reported for peppermint and commint (Niu et al., 1998; Kumar et al., 2009). The excised leaves upon infection with A. rhizogenes strain LBA 9402 started showing hairy roots from the wounded region after about 45 days from the day of inoculation. However the control explant became blackened and dried when transferred to phytohormone-free solid MS media with kanamycin (50 µg/ml). After 7 to 8 weeks of transformation with A. rhizogenes, putatively transformed hairy roots were subcultured and selected into solid MS media containing 50 µg/ml kanamycin (Figure 2). The transformed hairy roots showed vigorous ageotropic growth with lateral branching and root hairs after 9 to 11 weeks and their colour gradually changed from yellowish white to reddish brown.
Figure 2. Selection in antibiotic (a) Hairy roots started to appear from the explants; (b) and (c) branching of hairy roots; (d) and (e) browning of untransformed control explants.

Figure 3. A to C. Histochemical gus assay of hairy roots and formation of blue color; (A) and (B) transformed root lines (C) untransformed roots.

Beta-glucuronidase (gus) expression assay

From 13 kanamycin resistant root lines gus assay was performed for only 7 lines to determine their potential to react with gus substrate. The root of each lines obtained after 65 days was used for staining. Blue coloration was observed only in 5 lines and the intensity of coloration among the transgenic lines varied from each other. There was no blue colour development in untransformed roots (Figure 3). However, remaining 2 lines showed absence of gus expression and this could be due to rearrangement of the gus cassette during the transformation process.

PCR analysis for gus and nptII genes

The transformed root lines obtained were examined for the integration of vector T-DNA derived nptII and gus gene into the plant genome. For this, genomic DNA isolated from different putatively transformed hairy root lines were screened by PCR with gus and nptII gene specific primers. Five kanamycin resistant hairy root cultures assayed for the presence of partial sequence of gus and nptII gene showed an expected amplicon size of 171 and 153 bp, respectively (Figure 4). The results thus obtained showed correlation with morphology based identification of the putative hairy root lines and therefore provided an effective selection criterion for analysis of the transgene hairy root cultures taken in this study.

DISCUSSION

Plant transformation has become an active tool of research for the study of gene function and crop improvement nowadays. The development of a protocol that is rapid, efficient and cost effective for functional
Figure 4. PCR analysis of transformed lines for nptII (Lane 2, 4, 5, 6, 7), GUS (Lane 10, 12, 13, 14, 15), non-transformed lines as negative controls for nptII (Lane 3) GUS (Lane 9), 100 bp ladder (Lane 1 and 11), plasmid of pART27 and pBI121 as positive control for nptII (Lane 8) and GUS (16).

Table 2. Efficiency of transformation in A. rhizogenes with two different vectors.

| Construct | No. of explants | No. of roots developed | No. of roots after selection stress | Efficiency (%) |
|-----------|-----------------|------------------------|------------------------------------|----------------|
| pBI121    | 1260            | 24                     | 13                                 | 1.03           |
| pART27    | 1247            | 36                     | 15                                 | 1.2            |

Analysis of the gene would be useful. The use of A. rhizogenes mediated transformation is one of the approaches for understanding the gene function in plants. The results presented in this work supported the feasibility of A. rhizogenes mediated genetic transformation in C. assamica similar to the work demonstrated other dicots and monocots. The successful rate of transformation are dependent on several factors such as the type of tissue and plant genotype, concentration of bacterial culture, period of co-cultivation, the type of vector and selectable marker used (Gelvin, 2010; Citovsky et al., 2007). Therefore, finding the major factor for developing an efficient protocol is critical for any transformation work.

Generally use of acetosyringone for higher rate of transformation and enhanced hairy root formation has been suggested for Brassica oleracea (Henzi et al., 2000), Alhagi pseudoalhagi (Mei et al., 2001) tobacco (Kumar et al., 2006) and other plants which are recalcitrant in nature. In the present study we found that addition of acetosyringone to the co-cultivation medium and use of leaf as explant for agroinfection together with removal of excess Agrobacterium significantly enhanced the survival rate of transformants. We also evaluated the use of nptII (selection marker) and gus expression as a tool for monitoring the transformants and found that, among varying concentration of antibiotic used the best results were observed when selection was made with kanamycin 50 µg/ml for five weeks. During this time non-transformed tissues started browning and did not grow further, this phenomenon revealed localized death of plant cells at the site of injury and this may be due to hypersensitivity-like reaction functioning in the tissues. Also a very less number of transformants survived the selection process (Table 2), this suggests a sensitive reaction upon kanamycin selection which developed a necrotic response among the untransformed and enhanced the survival of tissues expressing nptII gene during cultivation. A significant variation in transient gus expression was observed in the present study and this could be due to influence of growth condition of the genotype examined or rearrangement of the gus cassette during the transformation process. Studies also suggest that differences in expression of genes that are closely related to the transformation process could also make differences in susceptibility to agrobacterium infection (Tie et al., 2012).

Therefore, it could be possible that the genes involved in different steps of the transformation event, failed to express that resulted in lower rate of transformation. In the present work it is observed that the best result were obtained with young growing tissues collected after kanamycin selection. Under these conditions gus expression was restricted to only few spots in the growing tissue. This suggests that use of strong promoter other than CaMV 35S could enhance gus expression and subsequently be taken as an effective criteria for selection of transformants in the genotype examined. Similar results were also obtained by workers where ubiquitin promoter have been shown to produce better
results than CaMV 35S for enhanced gus expression (Dong et al., 1991; Schledzewski and Mendel, 1994). So far, A. rhizogenes mediated genetic transformation system has been widely used as a powerful tool for comprehensive analyses of gene function and facilitating genetic improvement of crop plants. However there are few successful cases of A. rhizogenes mediated transformation in tea plant. In the present study, though transgenic hairy root system has been introduced in C. assamica using leaves infected by A. rhizogenes, LBA 9402, the overall transformation efficiency was low and requires further optimization. The present protocol provide foundation for transformation, selection and gene expression studies with two common visual marker, nptII and gus in C. assamica which may be practical for use in research and root functional genomics in particular.

Conclusion

In our crop genetic transformation has been successfully carried out using A. rhizogenes strain LBA 9402. Molecular and physicochemical analysis of the transformed roots showed effective transfer of foreign gene and its expression in highly branching tissue of tea hairy roots. Successful introduction of reporter gene in reporter gene and its expression in highly branching tissue of tea

Conflict of Interests

The authors have not declared any conflict of interests.

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