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GENETIC TRANSFORMATION AND HYBRIDIZATION

T. K. Mondal · A. Bhattacharya · P. S. Ahuja
P. K. Chand

Transgenic tea [*Camellia sinensis* (L.) O. Kuntze cv. Kangra Jat] plants obtained by *Agrobacterium*-mediated transformation of somatic embryos

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Abstract A protocol for the production of transgenic tea [*Camellia sinensis* (L.) O. Kuntze cv. Kangra Jat] was developed via *Agrobacterium*-mediated genetic transformation of somatic embryos. Two disarmed *Agrobacterium tumefaciens* strains, EHA 105 and LBA 4404, both carrying the binary plasmid p35SGUS-INT with the *npt*II gene and *gus*-intron were evaluated as vector systems. A number of parameters were tested with respect to maximizing transformation efficiency. While pre-culture, wounding and acetosyringone treatment were inhibitory, the bacterial growth phase (optical density; OD_{600} = 0.6), cell density (10^9/ml), co-cultivation period (5 days) and pH of the co-cultivation medium (5.6) had positive effects on transformation. Following co-cultivation, globular somatic embryos were placed on multiplication medium and stressed with kanamycin (50 µg/ml). Further selection occurred in the maturation and germination medium at an elevated kanamycin level (75 µg/ml). An average of 40% transient expression was evident based on the GUS histochemical assay. Kanamycin-resistant, GUS-positive embryos were germinated, and the resulting microshoots were multiplied in vitro. Integration of the transgenes into the tea nuclear genome was confirmed by PCR analysis using *npt*II- and *gus*-specific primers and by Southern hybridization using an *npt*II-specific probe. The transgenic shoots were micrografted onto seed-grown rootstocks of cv. Kangra Jat and eventually hardened in a walk-in polyhouse. This is the first report on the production of transgenic tea.

Keywords Tea (*Camellia sinensis*) · Somatic embryos · *Agrobacterium tumefaciens* · Woody plant transformation

Abbreviations *BA*: N^{6}-Benzyladenine · *IBA*: Indole-3-butyric acid · *gus*: β-Glucuronidase gene · *NAA*: α-Naphthaleneacetic acid · *nptII*: Neomycin phosphotransferase · *NOS*: Nopaline synthetase · *TDZ*: Thidiazuron

Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze], which belongs to the family Theaceae, is one of the most important woody plantation crops yielding a non-alcoholic beverage. Its long life cycle, self-incompatibility and high inbreeding depression limit its genetic improvement either via the selection of elites from existing natural populations or by the development of biclonal hybrids. In addition, a low fruit set and the difficulty of rooting vegetatively propagated cuttings in a nursery provide further constraints at every stage of the selection, hybridization and propagation in multi-location trials. Moreover, because of the non-availability of any biotic and/or abiotic stress-resistant mutants conventional breeding is limited to yield and quality parameters.

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 (Arokiaraj et al. 1998), "Royal Gala" apple (Liu et al. 1998), kiwifruit (Nakamura et al. 1999) and almond (Miguel and Oliveira 1999). A successful molecular breeding approach through *Agrobacterium*-mediated genetic transformation would represent a significant step in overcoming the existing constraints in

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T.K. Mondal
Biotechnology Laboratory, R & D Department, Tata Tea Ltd,
Munnar – 685612, Kerala, India

A. Bhattacharya · P.S. Ahuja
Biotechnology Division,
Institute of Himalayan Bioresource Technology, Palampur,
Kangra – 176-061, Himachal Pradesh, India

P.K. Chand (✉)
Plant Tissue & Cell Culture Facility,
Post-Graduate Department of Botany, Utkal University,
Bhubaneswar – 751 004, Orissa, India
e-mail: pkchanduubot@hotmail.com
Fax: +91-674-509293

tea improvement programmes. Unfortunately, there is no report to date on the genetic transformation of tea using *Agrobacterium tumefaciens*. Perhaps, one of the major limitations has been the lack of a suitable method for adventitious regeneration directly from explants. Therefore, the present study was aimed at exploiting an alternative plant regeneration system via somatic embryogenesis and optimizing several key factors to utilize tea somatic embryos in *Agrobacterium*-mediated genetic transformation.

Materials and methods

Induction and multiplication of somatic embryos

Mature green fruits were collected from tagged plants of a Chinary type of tea (*Camellia sinensis*), cv. Kangra Jat, growing in the Banuri Tea Garden at Palampur (Himachal Pradesh, India), which is situated at 32°N and 76°E and 1,230 m above sea level. After removing the fruit coat, we sorted the seeds by first soaking them in water for 2–3 h and selecting only the sinkers. The seeds were then washed thoroughly in Tween 80 for 5 min, followed by repeated rinses in distilled water (5–6 changes). Surface sterilization was carried out in a 4% (v/v) calcium hypochlorite solution for 10 min, followed by five to six rinses in autoclaved distilled water. The surface-sterilized seeds were germinated in half-strength MS basal salts (Murashige and Skoog 1962) medium containing 30 g/l sucrose and solidified with 0.7% (w/v) Difco agar (Qualigens, India). Green cotyledons were harvested from 15-day-old seedlings and their embryonic axes removed. Segments (0.5–1.0 cm long) of the de-embryonated cotyledons were inoculated in embryo induction medium (EIM) comprising half-strength MS salts supplemented with 20 g/l sucrose, 2.5 mg/l NAA and 0.2 mg/l BA. Following 6–8 weeks of culture, primary somatic embryos were multiplied in a modified MS medium (embryo multiplication medium, EMM) containing a reduced level of the nitrate salts of potassium (950 mg/l KNO_3) and ammonium (825 mg/l NH_4 NO_3) and fortified with 300 mg/l K_2SO_4, 2.0 mg/l BA, 0.2 mg/l IBA and 1.0 mg/l L-glutamine. The secondary embryos that formed were placed in maturation medium (MM) composed of MS + 40 g/l maltose + 3.0 g/l *trans*-cinnamic acid for 4 weeks, followed by transfer to germination medium (GM, MS + 1.5 mg/l GA_3) for 8 weeks. All cultures were maintained at 25°±2°C under a 12/12 h (day/night) photoperiod with light provided by cool-fluorescent tubes at a photon flux density of 52 µmol m^{-2} s^{-1}.

Bacterial strains and vector

Disarmed *A. tumefaciens* strains LBA 4404 (Hoekema et al. 1983) and EHA 105 (Hood et al. 1993), both carrying p35SGUSINT, were employed as vector systems for transformation. Plasmid p35SGUSINT (Vancanneyt et al. 1990) is a derivative of the binary vector pBin19 (Bevan 1984) and has (1) the NOS promoter-*npt*II-NOS terminator (polyA) cassette and (2) the *gus* reporter gene with a portable plant intron (*gus*-int) under the regulatory control of the CaMV 35S promoter and a CaMV-pA sequence at the left border (LB) (Fig. 1). For routine use, strains of *Agrobacterium* were grown in the dark at 28°C in agar-solidified YMB medium (Hooykas et al. 1977) supplemented with 50 µg/ml kanamycin monosulfate (Sigma, St. Louis, Mo.).

Determination of phytotoxic levels of selective and bactericidal antibiotics

Globular somatic embryos (2–3 mm) were inoculated in EMM augmented with different levels of kanamycin monosulfate (25, 30, 35, 40, 45, 50, 55, 60, 65 and 100 µg/ml). In a separate study, three bactericidal antibiotics, namely cephalexin (Sporidex; Ranbaxy India.), cefotaxime (Claforan; Russel India) and carbenicillin (Hi-media, India), were tested, each at different levels (200, 400, 600, 800 and 1,000 µg/ml). All antibiotics were filter-sterilized and added to the autoclaved medium after the latter had been cooled to 45°C prior to solidification. Each experiment had three replicates, each containing 30 somatic embryos. All experiments were repeated three times, and subculturing was carried out in the same medium at regular intervals of 15 days until the tissue became necrotic. Data were recorded with respect to the increase or decrease in the fresh weight of the somatic embryos, together with the extent of necrosis.

Transformation

Single bacterial colonies were prepared by serial dilutions, and each colony was then inoculated in liquid YMB medium (20 ml) containing 50 µg/ml kanamycin. The bacteria were then allowed to grow in the dark at 28°C for 16–18 h at 180 rpm. Bacterial cells corresponding to OD_{600}=0.6 were pelleted by centrifugation (6,000 rpm, 10 min) followed by washing twice with liquid YMB. The final cell density was adjusted to 10^9 cells/ml with liquid EMM. Somatic embryos were submerged in the bacterial suspension for 20 min, blot-dried on sterile filter paper and finally transferred to agar-solidified EMM. Following different durations of co-cultivation, the somatic embryos were washed twice with autoclaved distilled water, then subjected to two additional rinses with liquid EMM containing cephalexin (400 µg/ml), blot-dried and transferred to semi-solid EMM containing two bactericidal antibiotics (cephalexin and carbenicillin, 250 µg/ml each). Following 15 days of culture, somatic embryos were transferred to the same medium augmented with one bactericidal antibiotic (cephalexin or carbenicillin, 400 µg/ml) as well as the phytotoxic antibiotic kanamycin (50 µg/ml). Following a 8- to 12-week culture period, with subculturing to fresh medium at 15-day intervals, kanamycin-resistant somatic embryos were transferred to the maturation medium augmented with an elevated level of kanamycin (75 µg/ml) and, after 4 weeks, to the germination medium containing 75 µg/ml kanamycin.

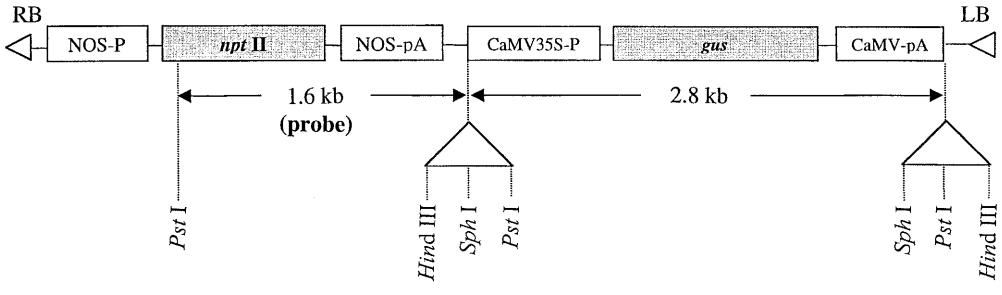

Fig. 1 Partial restriction map of T-DNA from p35SGUSINT indicating the position of the *npt*II probe (1.6-kb *Pst*I fragment)

Optimization of parameters

A range of parameters were evaluated, and each experiment included five replicates, each containing ten somatic embryos. The experiments were repeated four times each. These parameters included the length of the pre-culture period (0, 1, 2, 3, 4 days) of somatic embryos in EMM prior to infection, bacterial growth phase (OD values of 0.4, 0.6, 0.8, 1.0 at 600 nm), bacterial cell density (10^7, 10^8, 10^9, 10^{10} cells/ml), method of wounding of somatic embryos (abrasion with sterile sand/glass wool, pricking with hypodermic needle), length of co-cultivation period (0, 1, 2, 3, 4, 5, 6, 7 days), pH of the co-cultivation medium (EMM) (5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0) and acetosyringone concentration (10, 20, 30, 40, 50 µ*M*). The phenolic compound acetosyringone (Sigma-Aldrich) was dissolved in ethanol, and the stock volume made up in autoclaved distilled water. The appropriate aliquot of the filter-sterilized stock solution was added to autoclaved EMM to make the required final concentration. All of the parameters were evaluated and optimized on the basis of GUS activity of the somatic embryos.

Analysis of putative transformants

Assay of GUS activity

The histochemical assay of GUS activity was carried out as described by Jefferson et al. (1987). Somatic embryos or hand sections of young leaf tissue from the putative transgenic plants were soaked in GUS assay buffer in the wells of ELISA plates (Hi-media) and subjected to vacuum infiltration (1–2 min) in a vacuum desiccator until bubbles appeared. The ELISA plates were then sealed with Parafilm and incubated overnight at 37°C. Plant material was treated at 65°C with 70% ethanol twice at 1-h intervals to remove traces of chlorophyll. The possibility of endogenous GUS expression was tested by subjecting uninfected somatic embryos and tissues to the histochemical GUS assay. GUS expression was visually observed and photographed under a light stereo-zoom microscope (Nikon HF II).

Polymerase chain reaction characterization

DNA was isolated from young unfolded leaves of putative transformed and untransformed control plants using a CTAB procedure (Doyle and Doyle 1987). The forward and reverse primer (oligonucleotide) sequences (Bangalore Genei, India) used for PCR amplification of the *npt*II gene were 5′-CCA TCG GCT GCT CTG ATG CCG CCG T-3′ and 5′-AAG CGA TAG AAG GCG ATG GC TGC-3′, respectively, and those used for amplification of the *gus* gene were 5′-GGT GGG AAA GCG CGT TAC AAG-3′ and 5′-TGG ATC CCG GCA TAG TTA AA-3′. The primers were so designed as to give amplification products of the internal sequence of the *npt*II and *gus* genes of 693 bp and 650 bp, respectively. The optimum PCR mixture (25 µl) contained 1.5 U *Taq* DNA polymerase, 10 m*M* Tris-HCl (pH=9.0 at room temperature), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 10 pmol and 50 ng template DNA. We used 50 pg of p35SGUSINT plasmid DNA as a positive control. Each reaction was overlaid with 50 µl mineral oil to prevent evaporation. Samples for enzymatic amplification were subjected to an initial programme of one cycle of 95°C for 150 s, 60°C for 30 s, 72°C for 90 s followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 90 s. Finally, the reaction mixtures were allowed to complete an additional extension of 5 min at 72°C before rapid cooling to 4°C. The PCR was carried out using a Perkin Elmer DNA Thermal Cycler (Model 480) with the fastest available transitions between temperatures. The amplification products were separated by 1% agarose gel electrophoresis in 1× TAE buffer (0.04 *M* Tris-acetate, 1 m*M* EDTA, pH=8) at 80 V for 45 min and subsequently stained with ethidium bromide solution (0.5 µg/ml) for 10 min. The gel was destained in sterile distilled water (15 min) and photographed under a UV trans-illuminator (Fotodyne MP-ST) equipped with MP4 Polaroid Instant Camera System.

Southern hybridization

Southern blot analysis was according to Sambrook et al. (1989). Briefly, the method involved digesting 10 µg of leaf genomic DNA with *Pst*I, electrophoresing the digested products on a 0.8% agarose gel (25 V, overnight) and then blotting the DNA fragments onto nylon membrane (Hybond N^+; Amersham, Piscataway, N.J.). This was followed by hybridization with probe DNA that had been radiolabelled with α-[^{32}P]-dATP by random priming (BARC, Bombay, India). The probe which was prepared from the *E coli* plasmid p35SGUSINT was a 1.6-kb *Pst*I fragment containing the *npt*II gene (Fig. 1). The blots were first washed at low stringency (2× SSC, 0.15% SDS) twice at 65°C (30 min each), followed by two washes (30 min each) at moderate stringency (0.5× SSC, 0.1% SDS) at 65°C. They were then autoradiographed with an intensifying screen at –85°C for 5 days.

Establishment of transgenic plants

The transgenic microshoots were multiplied in vitro on MS medium containing 5 µ*M* TDZ and 10 µ*M* NAA (Mondal et al. 1998). These were micrografted onto the seedling-derived rootstocks of the same cultivar (Kangra Jat) according to the procedure described by Prakash et al. (1999). The grafted plants were grown in a walk-in polyhouse.

Results and discussion

Evaluation of the antibiotic effect on somatic embryos and *Agrobacterium*

A gradual reduction in the fresh weight of somatic embryos was associated with an increasing concentration of the phytotoxic antibiotic kanamycin. There was a total loss of chlorophyll pigmentation (bleaching) coupled with arrest in germination capacity at 35 µg/ml kanamycin. All somatic embryos were necrotic at 45 µg/ml kanamycin within 6–8 weeks. Kanamycin is generally used in the range of 30 µg/ml to 150 µg/ml for selecting transformed plant cells (Manders et al. 1994). In *Camellia*, 75 µg/ml was identified to be the lethal dose of kanamycin for in vitro internodes (Tosca et al. 1996). The optimum bactericidal concentration was 400 µg/ml regardless of the antibiotic used. Of the three bactericidal antibiotics we tested, cephalexin (400 µg/ml) had a negligible effect on the growth and organogenic response of the somatic embryos while being the most effective (followed by carbenicillin and cefotaxime) in controlling overgrowth of *Agrobacterium* (data not shown).

GUS activity of putative transformed somatic embryos and embryo-derived plants

Transient GUS activity was detected by characteristic blue spots which appeared on the somatic embryos 48 h following co-cultivation. Eventually this blue coloration

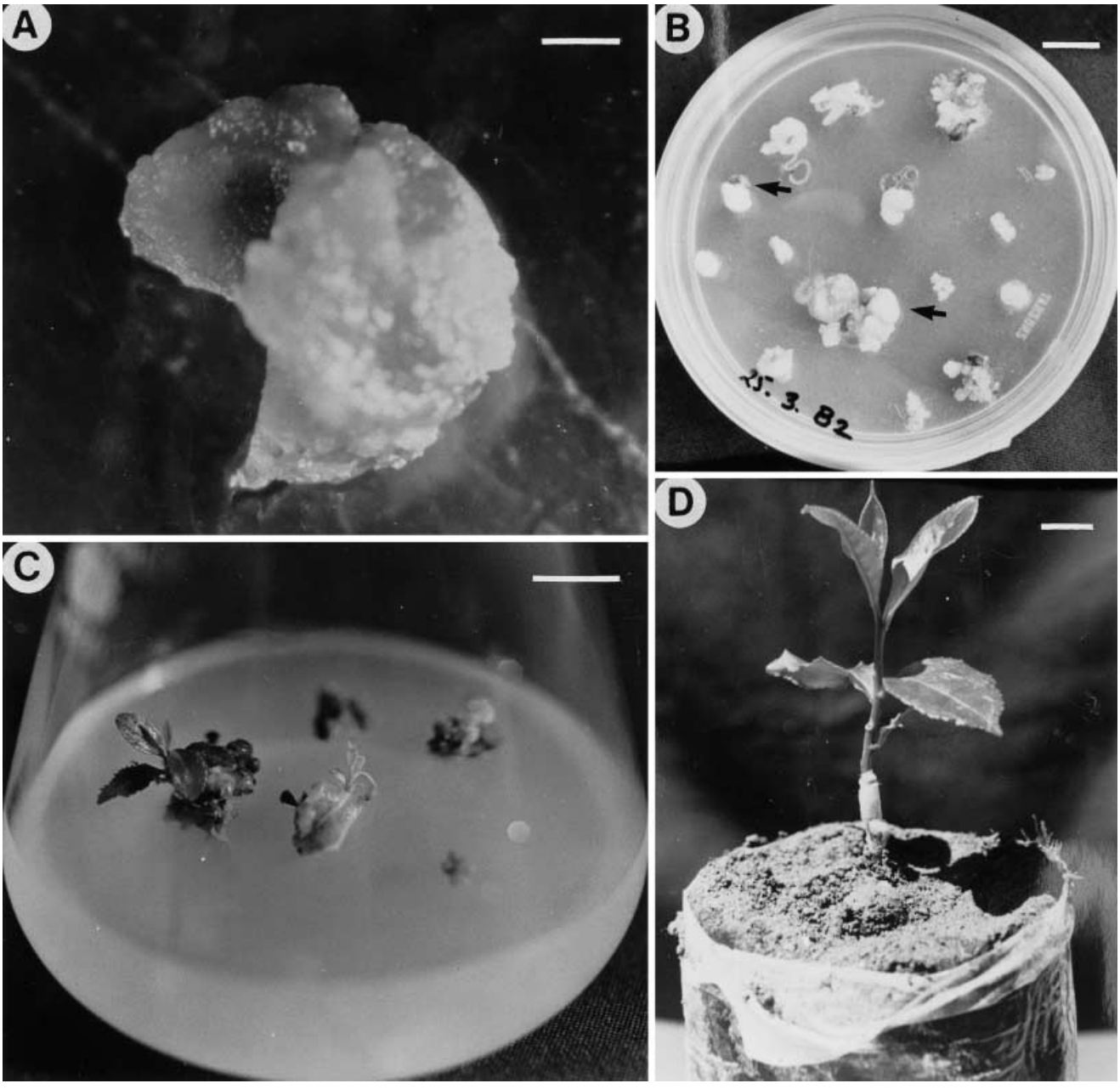

Fig. 2 **A** A GUS-positive somatic embryo 4 months following co-cultivation (*bar*: 3 mm). **B** Kanamycin selection of somatic embryos on embryo multiplication medium (EMM) + 250 µg/ml cephalexin + 50 µg/ml kanamycin (*bar*: 1 cm). **C** Kanamycin-resistant shoots from transformed embryos on germination medium (GM) + 75 µg/ml kanamycin (*bar*: 1 cm). **D** A transgenic shoot micrografted onto seedling-derived rootstock of cv. Kangra Jat and grown in a polyhouse (*bar:* 1 cm)

became uniformly distributed over all of the embryo surface (Fig. 2A). Putative transformed somatic embryos were selected, based on their growth on EMM augmented with the lethal dose of kanamycin (50 µg/ml) (Fig. 2B). Following successful growth on the maturation medium and, subsequently, on the germination medium, each supplemented with 75 µg/ml kanamycin, shoot regeneration occurred (Fig. 2C). A very strong GUS-positive signal was detected in the leaf tissues from 1-year-old shoots recovered through the germination of GUS-indexed kanamycin-resistant somatic embryos. No endogenous GUS expression was detectable in somatic embryos or tissues that had not undergone an *Agrobacterium* infection. The GUS-positive reaction of kanamycin-resistant somatic embryos or microshoots was unambiguously due to the expression of the transgene *gus* in the tissue and not due to the persisting *Agrobacterium*, which would, in fact, be unable to splice out the intron from the chimaeric *gus*-intron gene construct (p35SGUSINT, Vancanneyt et al. 1990) used in the transformation experiments. This construct has already been safely and successfully exploited in the *Agrobacterium*-mediated genetic transformation of several woody

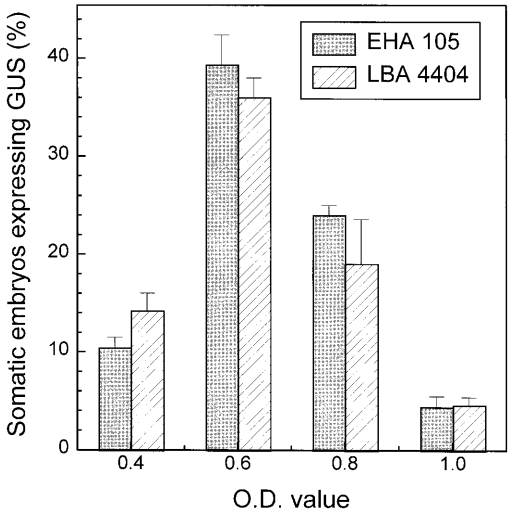

Fig. 3 Relationship between stages of growth of *Agrobacterium* culture and transformation efficiency

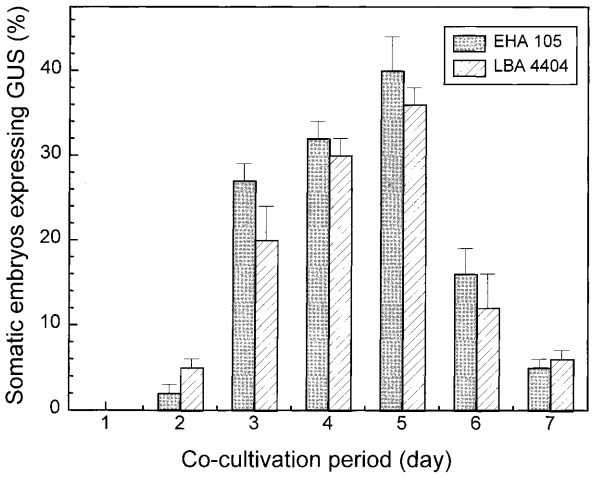

Fig. 5 Effect of duration of co-cultivation of somatic embryos with *Agrobacterium* on transformation efficiency

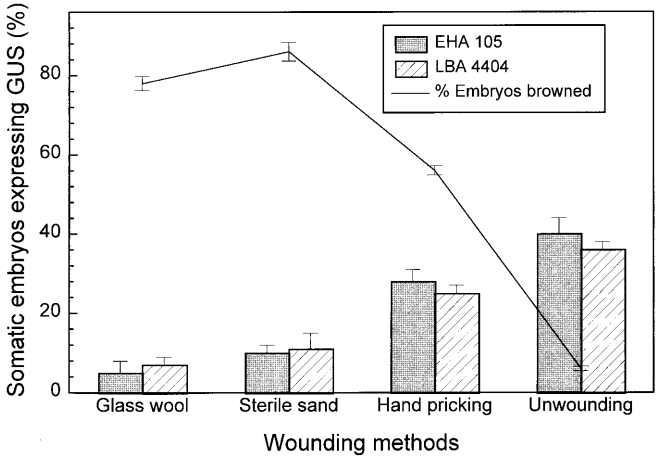

Fig. 4 Effect on the transformation efficiency of the different methods used for wounding the somatic embryos

tree species, including almond (*Prunus dulcis*; Miguel et al. 1999), "Royal Gala" apple (*Malus* sp.; Liu et al. 1998), citrange (*Citrus sinensis*; Cervera et al. 1998), lime orange (*Citrus aurantifolia*; Pena et al. 1997) and rubber (*Hevea brasiliensis*; Arokiaraj et al. 1998).

Optimization of transformation parameters

Pre-culture

Somatic embryos that were directly infected with *Agrobacterium* without pre-culture showed a higher transformation competence than the ones cultured in EMM prior to bacterial treatment. Transformation efficiency based on screening for GUS expression was 40–44% without pre-culture, while it varied from 5% to 18.3% for a 1- to -4-day pre-culture in EMM. The negative effect of explant pre-culture on woody plant transformation is well-documented. For example, in tamarillo, a 2-day pre-culture of leaf pieces reduced the transformation efficiency by 10% (Atkinson and Gardner 1993). Elimination of the pre-culture condition was also helpful in producing transformants in kiwifruit and apple (Janssen and Gardner 1993).

Bacterial growth phase and cell density

At a late log phase corresponding to OD_{600}=0.6, maximum transformation as measured by the GUS assay was achieved (Fig. 3), the efficiency of EHA 105 being higher (40%) than that of LBA 4404 (36%). An increase or decrease in the OD value resulted in a decrease in transformation efficiency. OD values greater than 0.8 were not suitable for transformation, and extensive tissue damage occurred at OD values greater than 1.0 due to bacterial overgrowth. At a higher OD, the regeneration of plant tissues in *Citrus* was inhibited by bacterial induced stress, and it also became difficult to control the overgrowth of bacteria following co-cultivation (Pena et al. 1995). A late log phase corresponding to an OD of 0.6 was the most effective for obtaining high rates of transformation in almond (Archilletti et al. 1995).

Of the range of bacterial cell densities tested at OD≅0.6, 10^9 cells/ml was the optimum. The requirement for an ideal bacterial density has been shown to vary with plant species. In *Citrus*, maximum transformation was achieved with 4×10^7 cells/ml (Pena et al. 1995), while in hybrid poplars 10^7 cfu/ml gave the maximum transformation efficiency (Howe et al. 1994). In the case of poplars, transformation frequencies varied greatly with the genotype as well as the *Agrobacterium* strain used (Confalonieri et al. 1997).

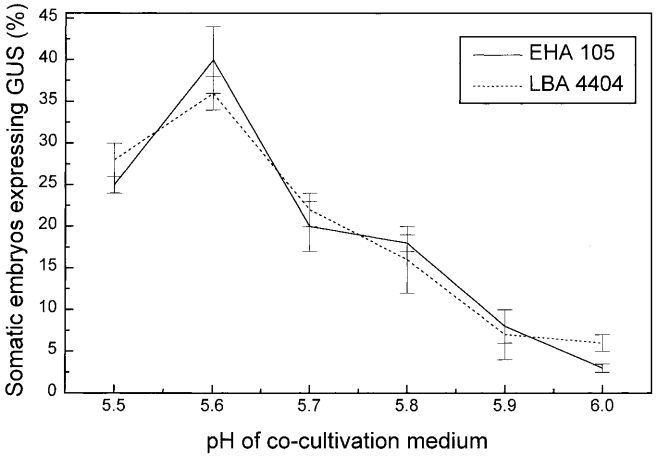

Fig. 6 Effect of pH of co-cultivation medium on the transformation efficiency

'Wounding' effect

The maximum number of transformation events (approx. 40%) were scored using intact (unwounded) somatic embryos (Fig. 4). When somatic embryos were injured with either glass wool or sterile sand, a lower number of transformation events (approx. 10%) coupled with a high rate of tissue browning were noticed. Thus, wounding was not only unnecessary for inducing transformation but also deleterious to tea somatic embryos. This was also found to be the case for walnut (McGranahan et al. 1988).

Co-cultivation period and pH

The co-cultivation of somatic embryos with either of the disarmed strains (EHA 105 or LBA 4404) for 5 days resulted in high-frequency transformation (Fig. 5). Co-cultivation for 2–7 days is generally considered to be suitable for *Agrobacterium*-mediated transformation, as reported for many plant species. More than 5 days of co-cultivation encouraged an overgrowth of bacteria with a concomitant decrease in transformation efficiency in garden pea (De Kathen and Jacobsen 1990) and flax (Dong and McHughen 1993). Co-cultivation for more than 3 days led to a decrease in transformation frequency and about 50% explant loss because of bacterial overgrowth in experiments designed to produce transgenic *Datura* (Ducrocq et al. 1994). The differential requirement of co-cultivation period largely depends upon the *Agrobacterium* strain used or the medium for bacterial culture or co-cultivation.

In the present study, co-cultivation medium with a pH of 5.6 yielded the highest number of transformation events (Fig. 6). The influence of the pH of the co-cultivation medium on transformation efficiency is well-documented in literature. Aliev et al. (1988) observed that the virulence of *A. tumefaciens* was related to the pH of the leaf and stem cell sap in cotton (*Gossypium hirsutum* cv. 109F), with the best infectivity being achieved at pH 5.5. In potato leaf discs a pH of 5.6 was the optimum for inciting the highest number of tumours when five different strains of *Agrobacterium* were tested (Boudjeniba and Hunault 1989). An acidic pH of 5.5 is generally considered to be suitable as acidic pHs may induce the *vir* (virulence) genes (Stachel et al. 1986; Al-Moerbe et al. 1988).

Acetosyringone treatment

The use of the phenolic inducer, acetosyringone, did not enhance the efficiency of transformation in the present study (data not shown). Similarly, in several other experiments on woody plants, particularly on plum (Mannie et al. 1991) and poplars (Confalonieri et al. 1997), acetosyringone did not help in increasing transformation efficiency. The suppression of transformation frequency by acetosyringone has also been reported in other varieties of poplars (De Kathen and Jacobsen 1990). The inability of acetosyringone to improve upon the transformation efficiency could be due to the inherent prevalence of high amounts of phenolics in woody plant tissues such as tea.

Molecular characterization of transformed plants

DNA obtained from several independent kanamycin-resistant, GUS-positive lines revealed the specific predicted amplification products of 693 bp and 650 bp with *npt*II- and *gus* gene-specific primers, respectively (Fig. 7). This indicated the presence of both the linked marker transgenes *npt*II and *gus* as a single T-DNA in the transformed genome. No amplification product was detected in DNA from untransformed shoots when subjected to PCR amplification with either of the two primers. The failure of some kanamycin-resistant transformants to produce any PCR band for either primer may indicate false positives ('escapes') through antibiotic selection despite an increase in the kanamycin concentration from 50 µg/ml to 75 µg/ml during subsequent stages of selection. 'Escape' is a major problem in woody plant transformation (Pena et al. 1995). Nevertheless, it is also important to strike a balance between the requirement for a high concentration of selective agent and its inhibitory effect on plant regeneration (Dong and McHughen 1993).

To demonstrate the stable transformation of tea plants with the *npt*II gene, we subjected five putative transgenic lines to Southern blot analysis. No homology was detected for control plant DNA as the *npt*II probe did not hybridize to genomic DNA from any of the tested plants regenerated from somatic embryos without an *Agrobacterium* infection. On the other hand, *Pst*I digestion of genomic DNA from each of the four putative transgenic lines generated an internal transgene fragment of 1.6 kb that hybridized to the *npt*II probe (Fig. 8). Additional

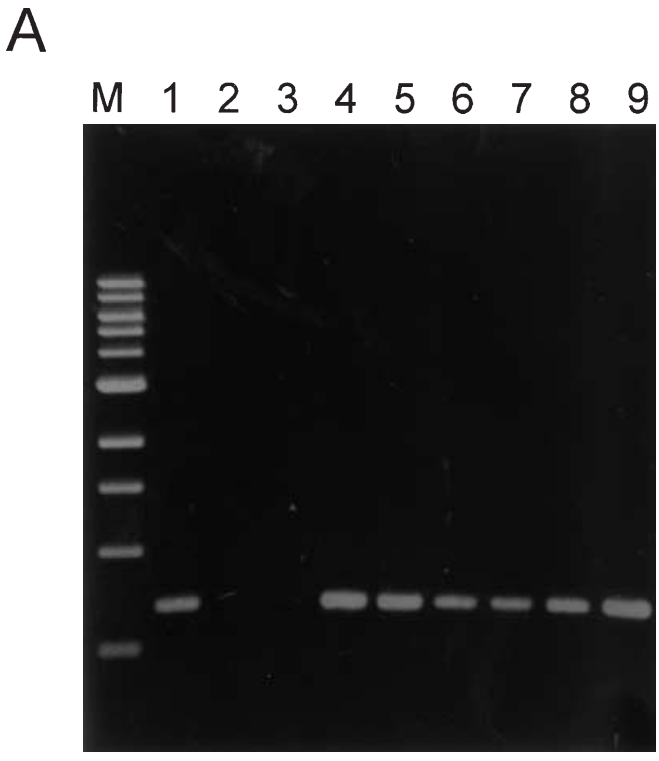

Fig. 7A,B PCR analysis to detect the presence of the *npt*II and *gus* genes in transgenic tea plants. **A** PCR amplification of the 693-bp fragment of the *npt*II gene. *Lanes*: *M* marker (1-kb ladder), *1* p35SGUSINT plasmid DNA (positive control), *2, 3* DNA from untransformed tea plants (negative control), *4–9* DNA from independently transformed plants. **B** PCR amplification of the 650-bp fragment of the *gus* gene. *Lanes*: *M* marker (100-bp ladder), *1* DNA from untransformed tea plant, *2–9* DNA from independently transformed plants

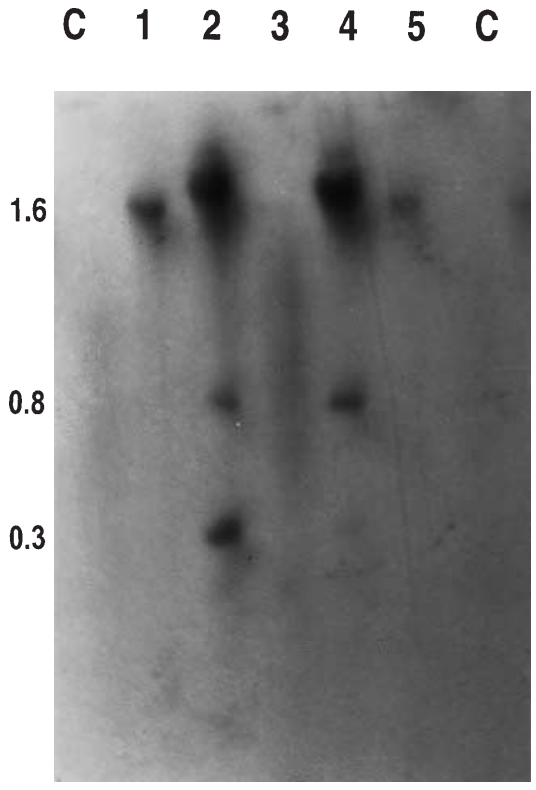

Fig. 8 Southern blot analysis of transgenic lines following the hybridization of *Pst*I-digested genomic DNA with the *npt*II probe. *Lanes*:*C* DNA from untransformed tea plant (control), *1–5* DNA from independently transformed plants

shorter fragments produced in some transgenic lines indicated that a deletion of part of the T-DNA containing *npt*II had occurred, perhaps during transformation or during plant regeneration. Nevertheless, the presence of variable size fragments provided evidence that these plants contained integrated copies of DNA sequences derived from the transgene from independent transformation events. The different banding patterns observed in the Southern blot could be due to multiple insertions, rearrangements and/or deletions of the integrated transgene in the regenerated plants, all of which are not uncommon in plants transformed with *Agrobacterium* vectors (Mercuri et al. 2000).

Only the microshoots from those putative transgenic lines which consistently showed predictably positive PCR amplification as well as Southern hybridization were micropropagated through nodal culture on MS augmented with 5 µ*M* TDZ and 10 µ*M* NAA. Following micrografting on seedling-grown rootstocks of cv. Kangra Jat about 80–90% of the micrografted transgenic shoots (Fig. 2D) survived in the polyhouse and developed into morphologically normal plants. The stability of transgene expression during development and its germ line transmission remains to be elucidated as it will take several years for these plants to flower and set fruit. Nevertheless, the protocol for producing transgenic tea plants that has been developed in the present study (Fig. 9)

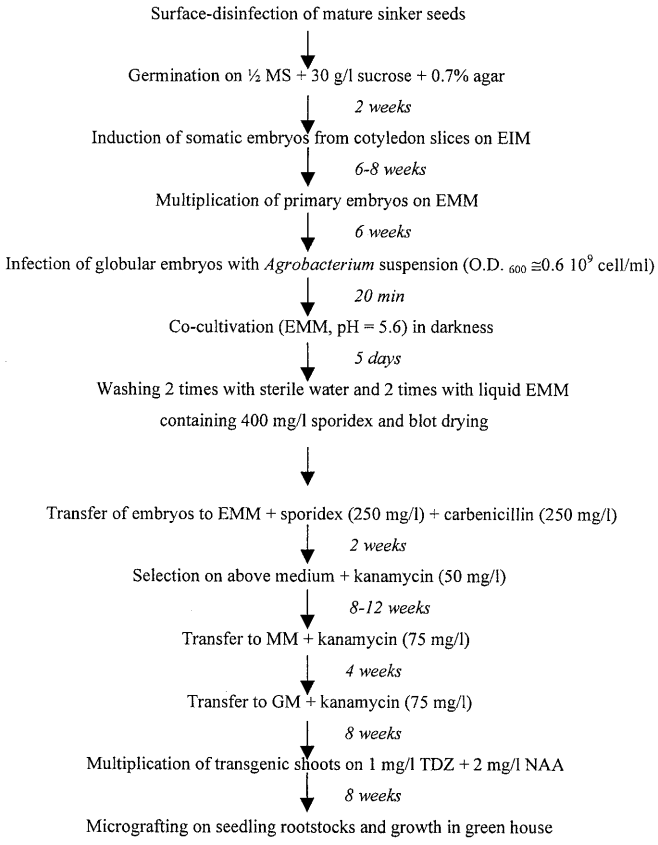

Fig. 9 The complete protocol for *Agrobacterium*–mediated genetic transformation of tea

should serve as an useful experimental basis for desirable gene introgression aiming at the genetic improvement of tea in the future.

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