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ORIGINAL ARTICLE

Development of transgenic tea plants from leaf explants by the biolistic gun method and their evaluation

Indra Sandal[1,2] · Rajash Koul[1] · Uksha Saini[1,3] · Mohina Mehta[1] · Nisha Dhiman[1,5] · Nitish Kumar[4] · Paramvir Singh Ahuja[1] · Amita Bhattacharya[1,5]

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Abstract The PDS1000-He biolistic gun was used to bombard plasmid DNA harbouring *gus* and *npt*II genes into tender young leaves of in vitro grown shoots of *Camellia sinensis* (tea). Out of a total of 500 bombarded leaves, 217 (43.4 %) showed callusing after 5 weeks on selection medium containing 1.71 µM kanamycin. Only 15 of these regenerated into indirect shoot buds. Only 7 out of 15 putative transformants showed the expected 400 bp signal with *gus* gene specific primers during PCR analysis. On the other hand, all the 15 putative transformants tested positive with *npt*II gene specific primers. In Southern hybridization with *npt*II specific gene probe, all the six randomly selected PCR positive plants showed stable integration of *npt*II gene. Both the transgenic and not-bombarded control plants showed phenotypic similarity under polyhouse conditions. Although their growth parameters were significantly at par, significantly lesser shoot height was recorded in transgenic plants. The reproductive behavior of the transgenics was also depressed. Thus, floral bud and flower abscission, fruit drop as well as empty seed production was higher in the transgenics as compared to control. Viability and germination of transgenic seeds was also significantly lower than control. Survival of the transgenic seedlings was also negligible (about 3 %). Hence, the chances of germline transmission of the transgenes were remarkably reduced in case of *gus*-transgenics. Tea being a vegetatively propagated plant, the method described in the present paper is an important approach for developing transgenics of elite tea plants from leaf explants.

Keywords *Camellia sinensis* · Flowering · Fruit · Leaf explants · Seeds · Transgenics

✉ Amita Bhattacharya
amitabhatta@ihbt.res.in

[1] Division of Biotechnology, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh 176061, India

[2] Division of Pulmonary Medicine, University of Utah School of Medicine, Salt Lake City, UT, USA

[3] Davis Heart and Lung Research Institute, Toledo, Columbus, Ohio, USA

[4] Centre for Biological Sciences (Biotechnology), School of Earth, Biological and Environmental Sciences, Central University of South Bihar, Patna, Bihar 800014, India

[5] Academy of Scientific and Innovative Research, New Delhi, India

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) of family Theaceae is an important beverage crop of the world. It is cultivated in several countries of Asia, Africa and South America as an important employment generator and a foreign exchange earner (Sandal et al. 2007). The beverage produced from tea leaves is rich in medicinal, nutritional, antimicrobial, anti-cancer and anti-aging properties (Chen 1999; Hara 2003; Cooper et al. 2005). Thus, the demand for tea leaves of superior quality is ever increasing. Yet, the amounts of quality tea actually produced are failing to meet the increasing demands. Biotic (fungi, insect pests and mites) and abiotic (frost, hail, chilling, drought, nutritional deficiencies) stresses cause major yield losses in tea (Bhattacharya and Ahuja 2001). Therefore, stress tolerance in tea continues to be an important goal of conventional breeding programs. However, tea breeding is constrained by long life cycle (6–10 years), high inbreeding depression,

self-incompatibility, low success rate of hand pollination and differences in the time of flowering and fruit bearing capacity of some clones (Mondal et al. 2004). Scarcity of land for tea improvement is another major constraint that cannot be ignored (Bhattacharya and Ahuja 2001). Alternatively, transgenic technology can serve as an attractive method for improving yield as well as quality or cup characteristics of tea (Bhattacharya et al. 2014; Song et al. 2014).

In this regard, transgenic tea plants were developed by the *Agrobacterium* as well as biolistic gun method of transformation in this laboratory (Mondal et al. 2001; Saini et al. 2012). The methods employed somatic embryos, derived from cotyledon explants of tea seeds. Seeds being the products of sexual reproduction are well known to be variable from each other. Thus, the use of seed explants requires labour intensive precautions such as maintenance of separate lines of somatic embryos derived from each seed (Saini et al. 2012). On the other hand, leaves and the selected elite mother plant from which they are plucked are true to type. Unlike seeds, all leaves of a plant retain the superior characteristics of its elite mother without any variation. Thus, when leaves are transformed with desirable genes, the superior traits of the mother plant are generally enhanced. This makes leaves more favoured as explants for genetic transformation as compared to cotyledons/seeds (Sandal et al. 2007). However, extensive optimization experiments are required before explants of a specific clone can become amenable to both regeneration and genetic transformation. In view of this, the aim of the present study was to optimize a biolistic gun method of transformation of tea leaf explants for the development of transgenic plants. The study also aimed at understanding the reproductive behavior of the transgenics with a view to generate primary information. The information is expected to serve as primary requisite for the control and maintenance of transgenic plants in the field and their commercial utilization.

Materials and methods

Plant material

Twenty year old bushes of Chinary type tea (*Camellia sinensis* (L.) O. Kuntze) growing in the Institute's Experimental Tea Farm at Banuri, Palampur, Himachal Pradesh, India (1292 m asl, 32.6°N and 78.19°E) were selected. Shoots were collected from these bushes during the seasons of fresh growth (March–May). Nodal segments (3.0 cm long) with a single axillary bud were excised from these shoots and surface sterilized by first washing in a solution of Tween-20 for 15 min followed by 0.04 % mercuric chloride for 5 min. After removing all traces of mercuric chloride by 4–5 rinses with sterile distilled water, the nodal segments (2.0 cm long after excision of exposed surfaces) were inoculated on 0.8 % agar (w/v) solidified half strength MS (Murashige and Skoog 1962) medium containing 3 % sucrose. After 2 weeks on this medium, the cultures were transferred to shoot multiplication medium (SMM) having full strength MS medium supplemented with 3 % sucrose, 8.88 μM 1-benzyl adenine (BA) and 0.98 μM indole 3-butyric acid (IBA) for further growth and multiplication. After three subcultures of 4 weeks each, completely folded first leaves (adjacent to the apical buds) were excised from the shoot cultures and used as explants for biolistic gun mediated genetic transformation. The pH of all the media were maintained at 5.8 prior to autoclaving and all the cultures were maintained at 25 ± 2 °C under a photoperiod of 16 h light (cool white lights with intensity of 52 μmol m^{-2} s^{-1}) and 8 h dark.

Bacterial strain and plasmid

The plasmid pRT99gus (Topfer et al. 1988) contained in *E. coli* is popularly used for standardization of protocols for biolistic mediated genetic transformation. The plasmid is a 6.71 kb pUC18 derivative containing the selection marker gene coding for neomycin phospho-transferase (NPTII) and *gusA* gene coding for β-glucuronidase (GUS). Both the genes are driven by the CaMV 35S promoter and are flanked by the NOS terminator (Fig. 1).

Plasmid DNA isolation

The alkaline lysis method of Sambrook et al. (1989) was employed to isolate plasmid DNA from fresh bacterial cultures that were grown overnight in Luria broth (LB) supplemented with 0.86 μM kanamycin monosulphate (Sigma-Aldrich, Bangalore, India). The isolated plasmid was purified using the purification columns (Qiagen, USA), and the presence of the *gus* and *npt*II genes in the isolated DNA were confirmed by colony PCR with gene specific primers. These comprised of (i) *npt*II: 5′-CCA-TCG-GCT–GCT-CTG-ATG-CCG–CCG-T-3′ (25 mer) as forward and 5′- AAG-CGA-TAG-AAG-GCG-ATG-CGC-TGC-3′ (24 mer) as reverse primers and also (ii) *gus*: 5′-GGT-GGG-AAA- GCG-CGT-TAC-AAG-3′ (21 mer) as forward and 5′-TGG-ATC-CCG-GCA-TAG-TTA-AA-3′ (20 mer) as reverse primers. The plasmid DNA was then used for biolistic mediated genetic transformation.

Pre-bombardment preparations

The pre-bombardment preparations were done in four steps namely, (1) sterilization of gold particles, (2) precipitation of plasmid DNA onto the gold particles (3) coating of

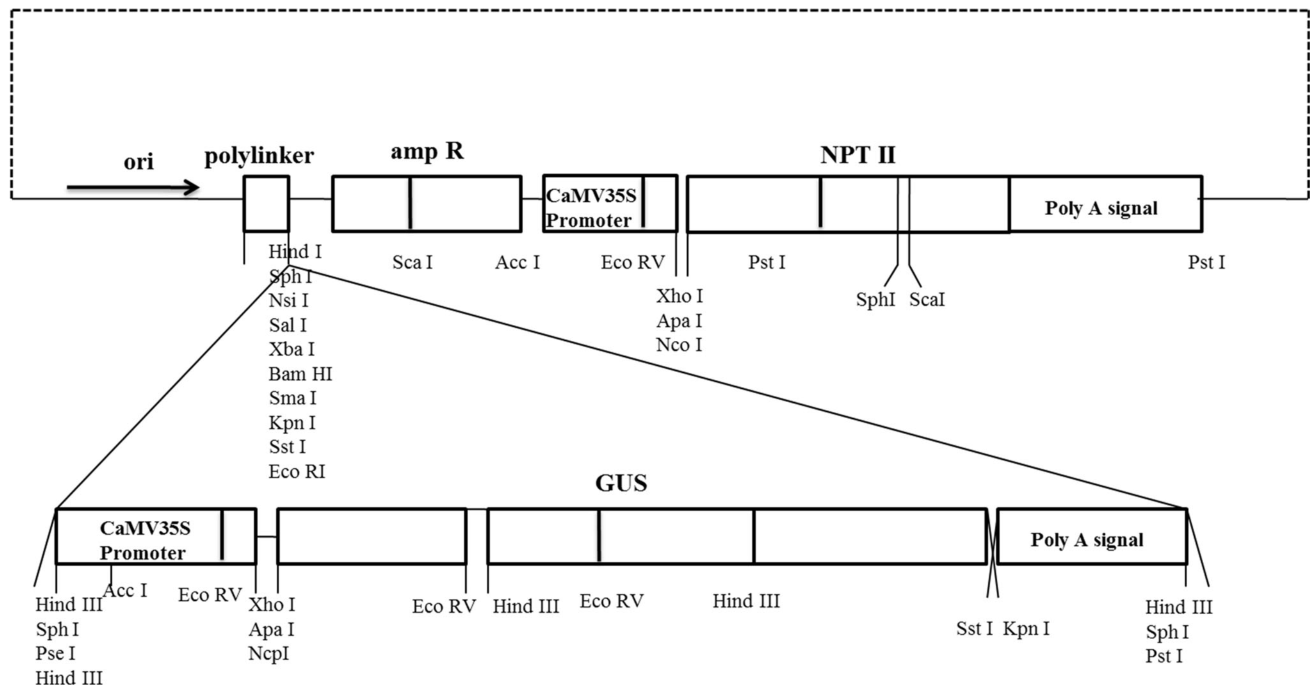

Fig. 1 The construct pRT99GUS harboring the genes *npt* II and *gus*A (encoding neomycin phosphotransferase and β-glucuronidase, respectively) under the constitutive control of CaMV 35S promoter

macrocarrier discs with plasmid DNA coated gold particles and finally (4) pretreatment and arrangement of the leaf explants on the medium for bombardment. Each step was performed as follows:

1. Gold particles of 1.0 μm (BioRad Laboratories Inc., CA, USA) were sterilized thrice in 70 % ethanol and finally once in absolute ethanol. For this, the gold particles were vortexed for 5 min in 1 ml of 70 % or absolute ethanol followed by incubation for 15 min. The gold particles were pelleted by microfugation and suspended in 1 ml of sterile de-ionized water. Finally, these were dispensed as 50 μl aliquots in eppendorfs and stored at 4 °C.
2. The 50 μl aliquots of gold particles were mixed with 1 μg μl^{-1} of plasmid DNA (10 μl volume), 10 μl of 2.5 M $CaCl_2$ and 10 μl of 0.5 M spermidine free of phosphate salts by vortexing for 3 min followed by brief centrifugation at 9000 rpm. The obtained pellet was suspended in absolute ethanol and centrifuged again. The final pellet comprising of the gold particles coated with plasmid DNA were suspended in 60 μl absolute ethanol.
3. 60 μl suspension of plasmid DNA coated gold particles was vortexed and 10 μl suspension was coated uniformly on each macro-carrier disc with the help of a pipette. The macro-carrier discs were always washed in 70 % ethanol and dried under laminar hood prior to use.
4. The leaf explants (average size of 1.2 × 0.8 cm) were immersed in PGR free liquid MS medium supplemented with 3 % sucrose (pH 5.8) for 4 h and then blotted on sterile filter paper. These were then inoculated on 0.8 % agar gelled MS medium supplemented with 3 % sucrose at same pH. Care was taken to inoculate the leaves closely at the centre of a 9.0 cm Petri plate with adaxial leaf surfaces touching the medium.

Bombardment of leaf explants

A helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) was used to bombard the leaves contained in each Petri plate twice. A macro-carrier disc coated with plasmid-DNA on gold particles was used for each bombardment. Based on previously optimized parameters of Sandal et al. (2006), the bombardment was performed under a chamber pressure of 94.8 kPa Hg at a 0.95 cm + 0.64 cm gap distance (the distance between the rupture disc and the macro-carrier), 16 mm macro-carrier flight distance (the distance between the macro-carrier and the stopping screen), 6 cm target distance (distance between stopping screen and target explant) and He burst pressure of 7584.23 kPa. Five Petri plates with 20 leaves in each were taken. For control, similar number of Petri plates each having 20 not-bombarded leaves were taken.

Regeneration and establishment of tissue culture plants from bombarded leaf explants

After 2 days in dark, the bombarded and not-bombarded control leaves were transferred to basal MS medium containing 0.8 % agar, 22.6 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 3 % sucrose and 1.71 µM kanamycin (MS1K) for 5 weeks. Following which, the leaf calli were transferred to 0.8 % agar semi solid basal MS medium containing 8.88 µM BA, 3 % sucrose and 1.71 µM kanamycin (MS2K) for the regeneration of adventitious shoot buds as per the modified method of Sandal et al. (2003). When the shoot buds of the putative transformants grew to a height of 2.0 cm after 12 weeks, they were excised and multiplied in liquid basal MS medium containing 5.0 µM thidiazuron (TDZ), 2 % sucrose and 1716.5 µM kanamycin monosulphate (MS3K). The putatively transformed shoots from each transformation event and the not-bombarded shoots (3.0 cm long) serving as control were maintained as independent lines and sub-culturing was done at regular interval of 4 weeks each. The not-bombarded control lines were also maintained for 5 weeks on basal MS medium containing 0.8 % agar, 22.6 µM 2, 4-D and 3 % sucrose (MS1), and then similarly on MS medium containing 0.8 % agar, 8.88 µM BA and 3 % sucrose (MS2) for adventitious shoot regeneration as per the modified method of Sandal et al. (2003). After three sub-cultures of 4 weeks each, both the not-bombarded and bombarded shoots were rooted, transferred to 12 inches pots containing sand: garden soil (1:1) and hardened as per the method described by Sharma et al. (1999). The plants were finally planted in soil under contained polyhouse conditions and maintained as tea bushes. The distances between each plant were 3 ft and that between each row was 2 ft.

Histochemical GUS assay

Histochemical GUS assay was performed in ten randomly selected leaves after 2 days of bombardment as per the method described by Jefferson (1987). Leaves from 4 year old transgenic plants were also assayed. In both the cases, the not-bombarded leaves served as control. The selected leaves were infiltrated with 100 mM X-gluc buffer (pH 7.0) containing 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 10 mM EDTA (pH 8.0), 0.1 % TritonX-100 and 1 mM of 5-bromo-3-chloro-2-indolyl-β, D-glucuronide sodium phosphate (Sigma Aldrich, Bangalore, India) under vacuum and incubated overnight at 37 °C in dark. The distinct indigo blue coloured scorable spots or sectors per explant were recorded and the percent GUS expression was calculated.

Molecular characterization of transgenic tea plants

DNA isolation

DNA was isolated from randomly chosen 15 independent transgenic shoots growing under in vitro conditions in MS3K supplemented with 1716.5 µM kanamycin. Plants growing under polyhouse conditions were also taken. Three not-bombarded lines growing in MS3K free of kanamycin as well as plants growing under polyhouse conditions served as control. The method of Doyle and Doyle (1990) was used to isolate genomic DNA from 1.0 g leaf tissues.

PCR analysis

The S1000 Thermal cycler (BioRad Laboratories (India) Pvt. Ltd., Gurgaon, India) was used to PCR amplify 50 ng of genomic DNA from 15 independent T0 transgenic and 3 lines of not-bombarded control. The plasmid DNA served as positive control. The amplification was done using *gus* and *npt*II specific forward and reverse primers by denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, annealing at 57 and 58 °C for 30 s and extension at 72 °C for 7 min followed by further 2 min. All PCR products were separated on 1.2 % agarose gel and photographed under a UV trans-illuminator (Fotodyne MP-St) equipped with MP4 Polaroid Instant Camera System). Amplification products of 400 bp were expected for each of the internal sequences of *gus* and *npt*II genes, respectively. The forward and reverse primers comprised of: (1) 5′-GGTGGGAAAGCGCGTTACAAG-3′ and 5′-TGGATCCCGGCATAGTTAAA-3′ for *gus* gene and (2) 5′-AGGATCTCCTGCATCTCAC-3′ and 5′-CCAAGCTCTTCAGCAATATC-3′ for *npt*II gene.

Southern analysis

Genomic DNA (30 µg) from the leaves of six independent lines of PCR positive T0 plants, two lines of T1 plants and one line each of not-bombarded controls of T0 and T1 plants were digested with *Hind*III (Invitrogen, Life Technologies Pvt. Ltd, Bengaluru, Karnataka, India). Digests were resolved on 0.8 % (w/v) agarose gels and blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences Little Chalfont, UK) under alkaline conditions. This was then hybridized with PCR-amplified *gus* and *npt*II gene probes after radiolabeling with α-^{32}P. For this, a mega-primer DNA labeling kit (Amersham, Biosciences, India) was used. The hybridized products were viewed on a phospho-imager and developed using X-Ray film (Kodak India Private Limited, India). Un-cut plasmid DNA served as control. Lighted up bands in each lane indicated transgene integration. In case of

T1 plants however, the *Hind*III digested genomic DNA was hybridized using biotin labeled *nptII* gene probe. In this case, the chromogenic substrate, alkaline phosphate conjugate streptavidin and NBT/BCIP-T were used to detect dark purple signals.

Growth and reproductive performance of transgenic tea plants under contained poly-house conditions

Phenotypic traits like branching, length of stem and internodes, leaf morphology, percentage flowering and seed set were recorded in ten plants each of transgenic and control plants at 4 weeks interval for four consecutive years. Percent germination of seeds from the transgenic and control plants followed by their seedling establishment and survival were also recorded.

Results

Raising of transgenic tea plants

The bombarded and not-bombarded leaves showed variable responses to 1.71 µM kanamycin (Table 1). Callusing was recorded in 65 % not-bombarded leaves after 5 weeks on MS1 free of kanamycin. When these were transferred to MS2, adventitious shoot buds developed (16.2 %) after 5 weeks. In case of MS1 K however, not-bombarded leaves (100 %) turned yellow and died, whereas, 217 out of a total of 500 bombarded leaves (43.4 %) showed callusing after 5 weeks on MS1 K (Fig. 2a, b). Adventitious shoot buds developed only in 15 out of a total of 217 (6.9 %) kanamycin resistant putatively transformed leaf calli after 6–7 weeks of transfer to MS2 K (Fig. 2c). These grew within 1–2 weeks (Fig. 2d).

Excised shoot buds derived from not-bombarded leaves died (100 %) after 15 weeks of transfer to liquid MS3K containing 1716.5 µM kanamycin, whereas, the putatively transformed shoots showed rapid multiplication and grew to a height of 3.0 cm (Fig. 2e). All the not-bombarded shoots (100 %) showed normal growth and multiplication in liquid SMM free of kanamycin (Fig. 2f). Root initiation was recorded in 83 and 89 % shoots derived from bombarded and not-bombarded leaves, respectively within 9–10 weeks of transfer to pots containing sand: garden soil. Even upon transfer to soil under polyhouse conditions, the hardened plants remained healthy (Fig. 2g) and continued to grow for 4 years into sturdy tea plants (Fig. 2h).

GUS expression

Transient GUS expression was evident from distinct indigo-blue coloured spots/sectors distributed over the surface of different leaf explants. These ranged from tiny to small spots and large patches. The spots/patches were recorded only in three out of ten putatively transformed leaf explants (Fig. 3a) and also in the leaves of seven out of 15 lines of 4 year old transgenics. Such expression was not observed in case of not-bombarded leaf explants (Fig. 3b).

Molecular characterization of transgenic tea plants

PCR analysis

Distinct bands corresponding to 400 bp of *gus* gene were detected in 7 (Fig. 4a) out of 15 lines of kanamycin resistant putative transformants in PCR. However, when *npt*II gene specific primers were used, the expected

Table 1 Summary showing the number of events produced and analyzed during each stage of genetic transformation

Treatments	Total number taken/tested	No. of responsive transformants derived after bombardment with		Control (not-bombarded)	
		Plasmid DNA containing *gus* and *npt*II genes	Linearized plasmid DNA minus *gus* and *npt*II genes	Kan−	Kan+
Bombarded leaf explants	800	500	100	100	100
Kanamycin resistance					
Callusing	800	217/500	0/100	65/100	0/100
Indirect shoot buds	317	15/217	0	16/100	0
GUS positive lines (in vitro)	40	10/10	0/10	0/10	0/10
GUS positive lines (plants in polyhouse)	35	7/15	0/10	0/10	0
PCR positive lines (for *gus*)	35	7/15	0/10	0/10	0
PCR positive lines (for *npt*II)	35	15/15	0/10	0/10	0
Southern positive T0 lines (for *npt*II)	5	4/4	0/0	0/1	0
Southern positive T1 lines (for *npt*II)	3	2/2	0/0	0/1	0

Fig. 2 In vitro plant regeneration from tea leaf explants. **a**, **b** Callusing on leaf explant, **c** initiation of adventitious shoot buds from leaf calli, **d** elongation of putatively transformed adventitious shoots on selection medium containing 1.71 µM kanamycin, **e** multiplication of putatively transformed shoots in liquid MS medium containing 5.0 µM TDZ, 2 % sucrose and 1716.5 µM kanamycin monosulphate, **f** multiplication of not-bombarded control shoots in kanamycin free liquid MS medium containing 5.0 µM TDZ and 2 % sucrose, **g** hardened transgenic plants growing in pots, **h** 4 year old transgenic tea bush growing under polyhouse. *Bars* in **a**–**d** = 1.4 cm, **e**, **f** = 3 cm, **g** = 20 cm and **h** = 60 cm

amplification product of 400 bp was observed in all the 15 kanamycin resistant lines (Fig. 4b). No amplification products were detected in the not-bombarded control lines (Fig. 4, lanes C, C1, C2 and C3), whereas, distinct bands corresponding to 400 bp were recorded in the positive control or plasmid DNA (Fig. 4, lane P).

Southern hybridization

Distinct but variable bands were observed in all the six randomly selected transgenic T0 plants (Fig. 5a). While a large 6.7 kb fragment was common to all the five transgenic lines, additional bands slightly larger than 6.7 kb were observed in each of lanes 1, 4, 5 and 6. Three bands, one higher than 6.7 kb, one of 6.7 kb and a band of about 3.1 kb were recorded in lane 1. A single band corresponding to 6.7 kb was recorded in lane 2 but a total of four bands of >6.7, 6.7, 2.5 and 1.8 kb were observed in lane 4. In lane 5 however, there were three bands of >6.7, 6.7 and <4.0 kb, while only two bands of >6.7 and 6.7 kb were recorded in lane 6. In case of lane 7, only a single band of 2.5 kb was recorded. However, no bands were detected in the not-bombarded control (Fig. 5b, lane C).

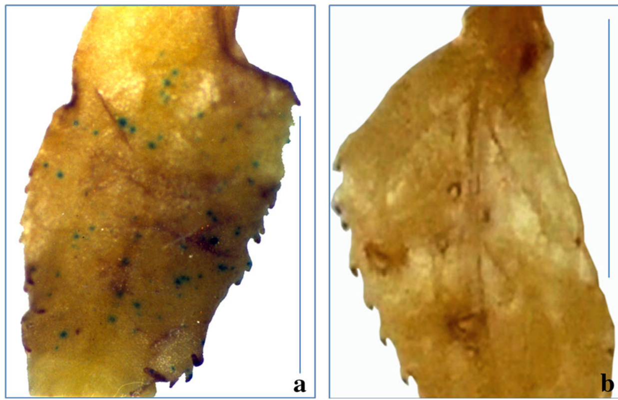

Fig. 3 Histochemical GUS assay of **a** putatively transformed leaf explant showing distinct indigo blue colour after bombardment with pRT99gus plasmid DNA, **b** not-bombarded control leaf explant. *Bars* = 0.7 cm

In case of independent T1 transgenic plants, lane 1 had two bands of size larger than 6.7 kb, whereas, lane 2 had a band higher and one corresponding to 6.7 kb (Fig. 5c). No bands were detected in lane C representing not-bombarded control.

Growth and reproductive performance of transgenic tea plants under poly-house conditions

The transgenic lines were phenotypically similar to the not-bombarded control plants both visually as well as statistically. Thus, both the transgenic and control plants had low spreading branches, thin and hardy stems and dull but dark green colored, oblong to ovate-oblong leaves with wavy margins. However, the shoot height of transgenics was significantly shorter than control (Table 2).

Under poly-house, both the transgenic and control plants produced a large number of flower buds throughout the year. Maximum number of flowering occurred when the mean diurnal temperature was above 20 °C i.e., during April to June (Fig. 6a, b). Continuous bud-abscission was also recorded in both transgenic and control plants but the rate of bud-abscission was invariably higher in the transgenic plants (Table 3). Many fully-opened transgenic flowers underwent drying and abscission (Fig. 6c), while only a small number of floral buds showed normal development (Fig. 6d1–5). The actual number of transgenic flower buds that opened during October to December (the peak season for fully opened flowers) was remarkably lower than control. While a small number of flowers also opened during January to March, and July to September, only a negligible number opened during April to June.

Fertilization (as indicated by flowers with withered petals but persistent style and stigma) was observed (Fig. 6d6–8) in all the seasons with exception to spring i.e., April to June. Maximum number of fruits were recorded during January to March, and the number in case of control plants was always higher than that of transgenics. Thus, in case of transgenic and control plants, 25 and 86 % of the fertilized ovaries produced fruits which abscised in high numbers i.e., 75 and 14 %, respectively (Fig. 6c, e, f). Fruit abscission in the transgenic plants was also accompanied by empty seeds or fruits with shrunken seeds (Fig. 6g). Finally, 7 and 47 %

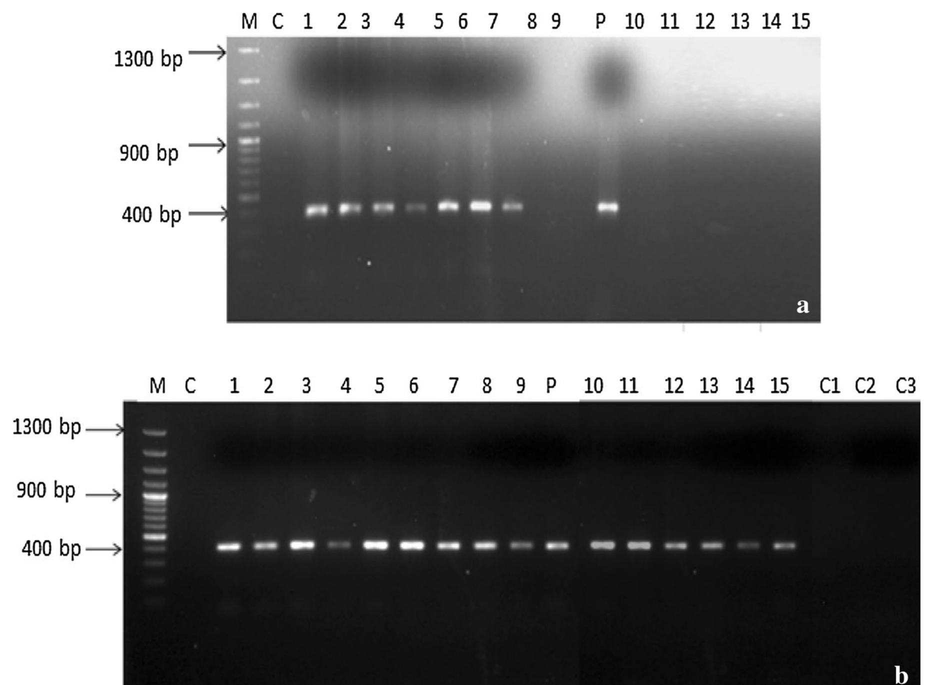

Fig. 4 PCR amplification of transgenic plants growing under poly-house condition showing the expected 400 bp amplification product of, **a** *gus* gene and **b** *npt*II gene where *lane M* 100 bp marker, *lane C*, *C1*, *C2*, *C3* untransformed control; *lane P* plasmid DNA as positive control; *lanes 1–15* independent transgenic plants

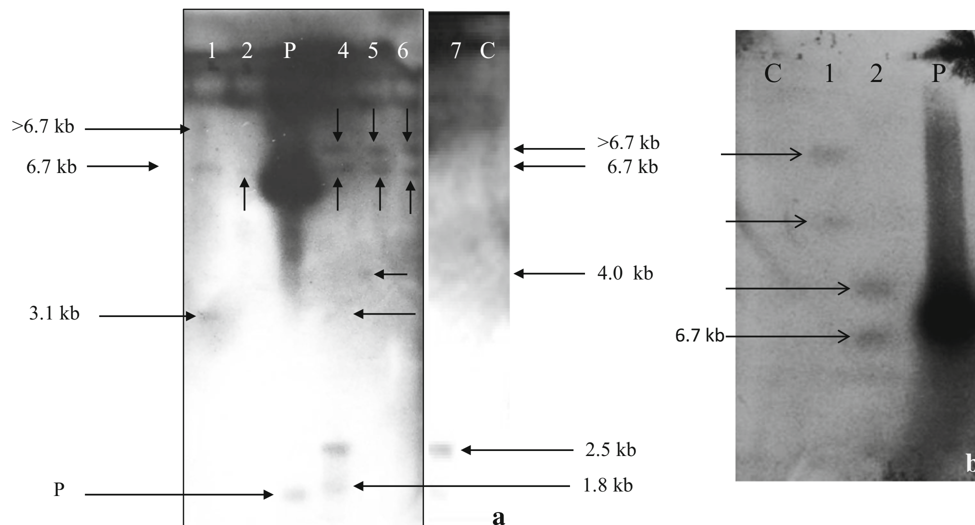

Fig. 5 Southern hybridization of genomic DNA digested with *Hind*III, **a** T0 transgenic where *lanes 1–7* independent transgenic lines, *lane C* not-bombarded control, *P* plasmid DNA as positive control, **b** T1 transgenic where *lanes 1–2* independent transgenic lines, *lane C* not-bombarded control, *lane P* plasmid DNA as positive control. A 1–8 kb marker was used

Table 2 Comparative analysis of growth parameters in transgenic and not-bombarded control plants

Plant type	Shoot height	No. of branches	Internode length	Leaf size
Control	$162.73^{a} \pm 11.13$	$7.67^{d} \pm 2.19$	$4.33^{d} \pm 0.32$	$29.00^{c} \pm 1.53$
Transgenic	$116.70^{b} \pm 6.17$	$9.67^{d} \pm 2.03$	$4.92^{d} \pm 0.17$	$26.67^{c} \pm 2.40$

Values are mean ± SEM (n = 10). Different lower-case letters in each row and column are significantly different at $P < 0.01$ in Duncan's multiple range test

germination was recorded in the normally developed seeds of transgenic and control, respectively (Fig. 6h, i). Although the number of transgenic seedlings that finally survived was significantly lower than not-bombarded control (Table 3), both the transgenic and control plantlets grew normally and were phenotypically similar.

Discussion

Somatic embryos derived from cotyledon explants of seeds have been generally used for the genetic transformation of tea (Mondal et al. 2001; Saini et al. 2012). However, the seeds are the products of sexual reproduction and are genetically variable from their mother plant. Therefore, they pose a threat of additional variability in the genome targeted for genetic modification. In contrast, leaf explants are more desirable (Sandal et al. 2007) because the leaves and the mother plant from which they are plucked are true to type. Leaves retain the superior characteristics of an elite mother without any variation. As a result, the elite characteristics of the mother plant are generally not lost when additional desirable transgenes are added into leaves through genetic transformation methods. Moreover, as compared to other explants, the higher surface area of the leaves further increases the ease of biolistic gun mediated transformation (Sandal et al. 2006). Despite these advantages, leaves were not used as explants for genetic transformation of tea in previous studies because of the lack of a reproducible regeneration system. In the present study however, the methods of Sandal et al. (2003, 2005, 2006) were modified and used to produce transgenic tea plants from leaf explants. As opposed to our earlier report, the step for rhizogenesis on medium containing BAP and IBA was omitted in the present study. This reduced the time frame for regenerating shoot buds and finally transgenic plants by almost 3–8 weeks. Although this time frame was higher than or at par with previous reports where somatic embryos were used (Mondal et al. 2001; Lopez et al. 2004; Saini et al. 2012), it was lower than that of previous report wherein leaves were used as explants (Sandal et al. 2006). Moreover in the present study, a target distance of 6 cm and a gap distance of 3/8 + ¼ inches were used to deliver plasmid DNA containing *gus* and *npt*II genes into a total of 500 tender in vitro leaves. In our earlier study, Saini et al. (2012) had optimized a target distance of 9 cm and gap distance of ¼ inches for the genetic transformation of somatic embryos. Often, optimization of parameters is necessary to avoid injury as well as undesirable transgene integration in delicate tissues (Birch and Franks 1991; Jagga-Chugh et al. 2012; Kim et al. 2012). Optimization of bombardment parameters has often resulted in the production of

Fig. 6 Reproductive behaviour in 4 year old not-bombarded control and transgenic plants growing under polyhouse condition. **a** Profuse flowering and flower abscission in transgenic, **b** a fully opened normal flower, **c** desiccation of flowers and necrosis of fertilized ovaries in transgenic, **d** stages of fruit/seed formation in control plants where *1–3* bud growth; *4* half opened flower; *5* fully opened flower; *6* fertilization; *7–8* fertilized ovary; *9–10* fruit development; *11* dehisced fruit with mature seeds, **e** abscission and necrosis at each stage of fruit/seed development in transgenic where *1* flower; *2–3* fertilized ovary; *4* dehisced fruit with under-developed dried seeds and fruits, **f** high rate of necrosis of fertilized ovaries in transgenic, **g** aborted transgenic seeds, **h** dehisced not-bombarded control fruit with mature and viable seeds, **i** transgenic seedling. *Bars* in **a** = 30 cm, **b**, **c** = 2 cm, **d**, **e** = 2 cm, **f** = 0.5 cm, **g**, **h** = 1.5 cm and **i** = 5 cm

Table 3 Comparison of the reproductive behavior of transgenic and not-bombarded control plants

Traits studied	Percent response	
	Transgenic	Not-bombarded control
Floral bud abscission	48.2 ± 3.26^{c}	25.0 ± 2.89^{de}
Fertilized ovaries (withered petals but green persistent style) that developed into fruits	25.2 ± 1.25^{de}	86.2 ± 0.97^{a}
Fruit abscission	75.2 ± 4.29^{ab}	14.4 ± 3.7^{ef}
Viable and full seeds	13.6 ± 3.19^{cd}	63.2 ± 3.6^{b}
Seed germination	7.0 ± 4.3^{f}	47.2 ± 2.7^{c}
Seedling survival	2.6 ± 1.24^{f}	47.4 ± 3.0^{c}

Values are mean ± SeM (n = 10). Different lower-case letters in each row and column are significantly different at $P < 0.01$ in Duncan's multiple range test

several commercially important tropical crops (De Guglielmo-Cróquer et al. 2010; Saini et al. 2012; Bhattacharyya et al. 2015).

Out of the 500 leaf explants that were bombarded, only 43.4 % (217) were resistant to 1.71 µM kanamycin and developed further to produce 15 independently transformed

putative adventitious shoots (Fig. 2c–e). When these were subjected to PCR analysis, all the lines tested positive for *npt*II, whereas, only 7 out of 15 transformants amplified the expected band for *gus* gene (Table 1; Fig. 4a). The fact that this was not an outcome of failed PCR reaction was evident from the distinct band obtained in case of positive control or plasmid DNA (Fig. 4a, lane P). This indicated that the *gus* transgene was perhaps either truncated, deleted or rearranged in 8 out of the 15 lines during transformation and/or plant regeneration. Transgene rearrangement and breakage are commonly associated with biolistic mediated transformation (Wilson et al. 2006; Chen et al. 2007; Kim et al. 2012; Tassy et al. 2014). Thus, direct methods of transgene delivery and integration often lead to chimerism in the target genome (Christou 1990). Chimerism is reported to persist in tissues of several herbaceous and woody plants even after rigorous selection of transformants (Bhat and Srinivasan 2002; Dalla Costa et al. 2014). It is implicated to result from transient expression of transgene(s) during the early stages of in vitro plant regeneration (Faize et al. 2010). In the present study, GUS expression was evident in the form of indigo blue spots to large sectors in almost all the in vitro leaves assayed after 2 days of bombardment, whereas, no GUS expression was recorded in the leaves of 8 out of 15 lines of 4 year old transgenics. This, thereby indicated the transient expression of *gus* gene in these lines. The results of histochemical GUS assay of the leaves of 4 year old plants also corresponded with PCR results when *gus* gene specific primers were used (Fig. 4a).

Despite the above observations, stable integration of *nptII* gene in tea genome was evident from the fact that all the randomly selected lines tested positive in both PCR and Southern hybridization with *npt*II gene specific primers and probe, respectively. Finally, a total of 15 true transgenic lines for *npt*II were generated (Fig. 4b) and multiplied as individual lines. In previous reports of Mondal et al. (2001) and Sandal et al. (2006, 2007), the actual number of Southern positive transgenic lines and final stable transformation efficiency were not mentioned. In the present study however, all the six lines tested positive in Southern hybridization. A similar observation was reported by Lopez et al. (2004) and Saini et al. (2012).

Transgene re-arrangements are known to alter the phenotype, the growth behavior or the trait for which a particular crop is useful or beneficial (Wilson et al. 2006). Although rearrangement of the integrated transgene was evident in the transgenic tea plants developed in the present study, yet their phenotypic characteristics remained largely unaltered with exception to lesser height of transgenics as compared to control (Table 2). While there was no mention of the phenotypic traits of the transgenics generated through *Agrobacterium* mediated transformation of leaf explants (Sandal et al. 2007), all previous reports described the transgenic lines generated from somatic embryos to be normal (Mondal et al. 2001; Lopez et al. 2004; Saini et al. 2012). While none of the previous studies reported about the reproductive behavior of the transgenic plants generated by them, distinct differences in the reproductive behavior of the transgenics and control plants were observed in the present study (Table 3). Higher rates of floral bud and fruit abscission in the transgenics were accompanied by the production of lower number of normal and viable seeds as compared to control. Finally, normal germination was recorded only in 7 % transgenic seeds as opposed to 47 % in not-bombarded control seeds. In case of transgenics, only 2.6 % seedlings finally survived to grow into normal plants. Thus, a marked suppression in the reproductive capacity was evident in the transgenics. Besides a strong possibility of sterile gamete(s) formation, such suppression could be the result of one or more of the different factors such as position effect of integrated transgene, homology dependent silencing of endogenous genes, genetic and/or epigenetic changes induced during the process of in vitro culturing and genetic transformation (Curtis et al. 2000; Aragão et al. 1996). However, the observed reproductive behavior could also be specific to the ten transgenic tea plants that were analysed in the present study.

Suppression of reproductive behavior in tea is advantageous because it can reduce the chances of transgene outflow. Adding to the benefit is the fact that germ-line transmission in tea is automatically prevented as it is propagated vegetatively. In this regard, the present work will prove useful for stacking genes of important agricultural traits and quality parameters into selected elite plants of tea.

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