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GENETIC TRANSFORMATION AND HYBRIDIZATION

Agrobacterium-mediated genetic transformation of tea leaf explants: effects of counteracting bactericidity of leaf polyphenols without loss of bacterial virulence

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Abstract Tea is one of the major crops in Asia and Africa, and its improvement by genetic modification is important for economy of many tea-producing regions. Although somatic embryos derived from cotyledon explants have been transformed with *Agrobacterium*, the leaves of several commercially important tea cultivars have remained recalcitrant to transformation, largely due to bactericidal effect of polyphenols that are exuded by tea leaves *in vitro*. Moreover, the commonly used polyphenol adsorbents and antioxidants cannot overcome this problem. Leaf explants, however, are more desirable than cotyledon-derived somatic embryos, especially when it is necessary to further improve a selected elite and also retain its superior traits. Thus, we developed a procedure for *Agrobacterium*-mediated genetic transformation of tea leaf explants which is based on the presence of L-glutamine in the co-cultivation medium. We then showed that the transformation process is facilitated via a protective action of L-glutamine against bactericidal effects of leaf polyphenols without affecting the bacterial virulence (*vir*) gene expression.

Keywords *Agrobacterium tumefaciens* · Bactericidal polyphenols · Genetic transformation · L-Glutamine · Leaf explants · Tea

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Abbreviations AS: Acetosyringone · BM: Basal Murashige and Skoog's medium · SEM: Secondary somatic embryogenesis medium · SRM: Shoot regeneration medium from leaf explants · 2,4-D: 2,4-Dichlorophenoxyacetic acid · YMB: Yeast mannitol broth · LB: Luria Bertani medium

Introduction

More than 3 decades after the discovery of the transforming ability of *Agrobacterium* (Chilton et al. 1977), *Agrobacterium*-mediated genetic transformation is still the most widely used method of producing transgenic plants (Dunwell 2000). Besides being cheaper and simpler than most direct gene transfer methods, it allows little rearrangement of transgenes, and efficient integration of the transgene into the plant genome (Ingelbrecht et al. 1991). Thus, it is not surprising that a large number of crop plants (Curtis 2004) have been transformed using this method. In the case of tea, one of the major crops in Asia and Africa, somatic embryos derived from cotyledon explants have been transformed with *Agrobacterium* harboring *gus* and *npt*II genes (Mondal et al. 2002; Lopez et al. 2004); however, the leaves of several commercially important tea cultivars have remained largely recalcitrant to transformation. This is mainly due to the exudation of high contents of bactericidal polyphenols (Biao 1998; Fukai et al. 1991; Kumar et al. 2004) and lack of suitable regeneration systems (Matsumoto and Fukui 1998, 1999; Mondal et al. 2004).

The leaf explants are more desirable than cotyledon-derived somatic embryos, especially when it is necessary to further improve a selected elite and also retain its superior traits. Genetic transformation of leaf explants enables one to introduce the genes of interest for better adaptation, quality

and yield into the selected plant without losing the clonal fidelity. In this regard, the recent success of Sandal et al. (2005) in regenerating shoots from leaf explants of Kangra jat cultivar in tea via a callus phase opened up the possibility of using leaf explants of this cultivar for *Agrobacterium*-mediated genetic transformation, provided the initial block in inducing *Agrobacterium* infection was overcome. To this end, it was necessary to counteract the bactericidal effect of polyphenols that are exuded by tea leaves *in vitro*, especially because the commonly used polyphenol adsorbents (e.g., PVP and charcoal) and antioxidants (e.g., cysteine, ascorbic acid and glutathione) failed to do so (Kumar 2003). Our observations indicate that L-glutamine, when incorporated into the culture medium, besides retaining the regeneration potential of the tea leaves, allows *Agrobacterium* infection. This is consistent with earlier reports of the use of a modified MS medium containing L-glutamine for the production of transgenic tea plants from somatic embryos (Mondal et al. 2002). Here, we report that co-cultivation media supplemented with L-glutamine at specific concentrations allow *Agrobacterium*-mediated genetic transformation of leaf explants of Kangra jat cultivar of tea via protection against bactericidal effects of leaf polyphenols without affecting the bacterial virulence (*vir*) gene expression.

Materials and methods

Culture initiation

Aseptic cultures of nodal segments were established as described by Sandal et al. (2005) from selected elite plants of Chinary type tea (*Camellia sinensis* L. O. Kuntze) cv. Kangra jat growing in the Tea Experimental Farm, IHBT, Palampur (1290 m above mean sea level, 32.6 N and 78.18 E). After three subcultures of 4 weeks each, the first leaves (i.e., the leaf subtending the apical bud), which have been reported to have good shoot regeneration potential (Sandal et al. 2005), were used as explants for *Agrobacterium*-mediated genetic transformation.

Bacterial strain and plasmid

We used a disarmed *Agrobacterium tumefaciens* strain EHA105 carrying p35SGUSINT (Vancanneyt et al. 1990), which is a derivative of the binary vector pBin10 and has a NOS promoter-*hpt*-NOS terminator (polyA) cassette and a *gus* reporter gene with a plant intron (*gus*-int) under the control of CaMV35S promoter. The T-DNA region of the p35SGUSINT-based plasmid used in our experiments is shown in Fig. 1A.

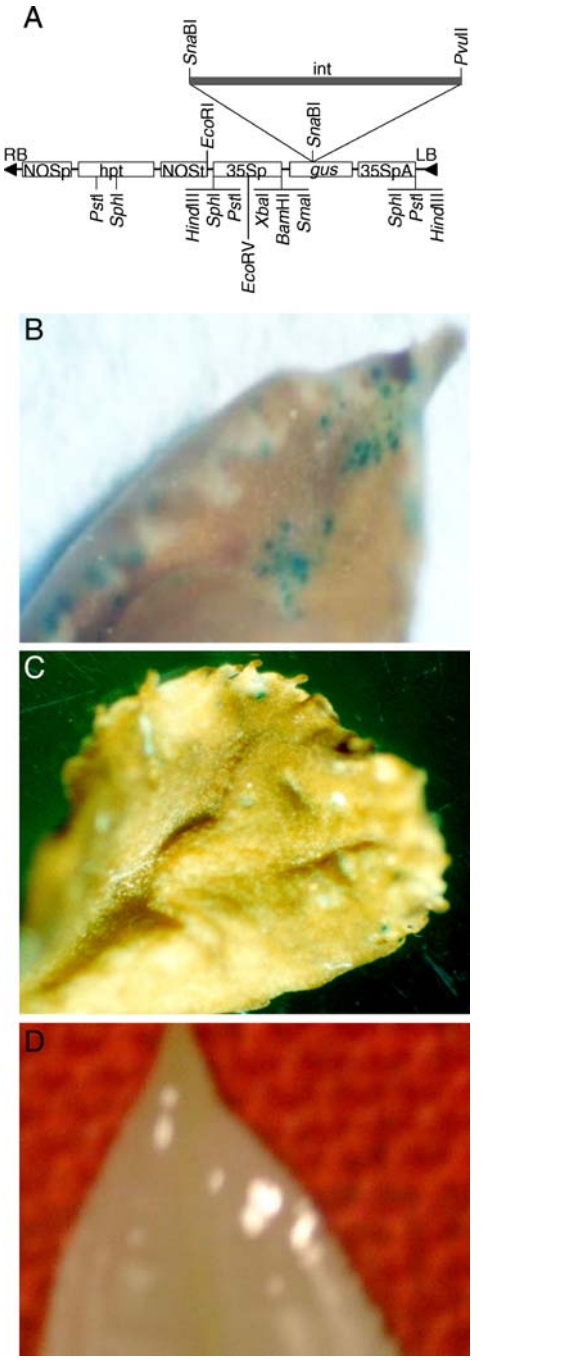

Fig 1 Transient expression of GUS activity contained within *Agrobacterium* T-DNA in tea leaf explants. **A** A schematic map of the T-region of the p35SGUSINT-based plasmid carrying the *gus*-int gene. RB and LB, right and left T-DNA borders, respectively; NOSp and NOSt, nopaline synthase promoter and terminator, respectively; 35Sp and 35SpA, promoter and polyA signal, respectively, of the 35S RNA polymerase gene of *Cauliflower mosaic virus*. The intron (int) is shown as a SnaBI-PvuII insert into the *gus* gene. **B**, **C** Two independent leaf explants co-cultivated with *Agrobacterium*. **D** Untransformed, control leaf explant

Media composition and transformation

Bacterial cells grown to optical density $A_{600} = 0.6$ were pelleted by centrifugation at 6000 rpm, from fresh overnight grown cultures in liquid yeast mannitol broth (YMB) containing 25 μg/ml hygromycin. The bacterial pellet was resuspended in fresh YMB to an optimal cell density of 10^9 cells/ml (Mondal et al. 2002). The leaf explants were submerged in the resulting bacterial culture for 20 min, blotted on sterile filter paper to remove excess *Agrobacteria* and then transferred to solid agar co-cultivation media of different pH values (pH 5.2, 5.4, 5.6, and 5.8) supplemented with L-glutamine (0.5, 1.0, and 2.0 g/l calculated prior to autoclaving). Note that, for best results, L-glutamine should be autoclaved and not filter-sterilized.

Other media, i.e., the hormone-free basal Murashige and Skoog's medium (Murashige and Skoog 1962) (BM), modified medium of Mondal et al. (2001) for secondary somatic embryogenesis (SEM), and shoot regeneration medium (SRM) of Sandal et al. (2005), with and without L-glutamine were also tested. For negative control, the leaves were submerged in liquid YMB without bacteria. The leaves were co-cultivated with *Agrobacterium* in the tested media for 5 days at different temperatures (19, 22, 25, and 28°C), washed in liquid SRM containing cefotaxime (400 μg/ml) to remove *Agrobacterium*, blotted on filter paper and transferred to selection medium containing 10 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 50 μg/ml hygromycin and 400 μg/ml cefotaxime for 1 week followed by their transfer to a cefotaxime-free selection medium. After 6 weeks, proliferating calli resistant to hygromycin were transferred to hygromycin-free shoot regeneration medium, and tissues from putative transformants were analyzed for transgene integration. All experiments were repeated twice and each of them included five replicates with 10 leaves per culture dish.

Histochemical GUS staining

GUS expression was detected 5 days after co-cultivation by vacuum-infiltrating the randomly selected transformed leaves with the GUS assay buffer as described (Jefferson 1987) using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; BioVectra, PEI, Canada) as substrate. GUS expression was scored as number of indigo-blue areas detected under a Nikon HF II light stereo-zoom microscope.

PCR analysis of transformants

Six weeks after transferring to the selection medium, total genomic DNA was extracted from 500 mg of randomly selected leaf calli as described by Doyle and Doyle (1990) and analyzed for the presence of the *gus*-int transgene by PCR amplification. The forward and reverse primers used for amplification of the *gus*-int sequences were 5′-GGTGGGAAAGCGCGTTACAAG-3′ and 5′-TGGATCCCGGCATAGTTAAA-3′, respectively (Bangalore, Genei, India), and they were designed as to amplify a ~600-bp fragment of the T-DNA region of p35SGUSINT (Vancanneyt et al. 1990) containing 412 bp of the internal sequence of the *gus* gene and the 189-bp intron. A total of ~50 ng of genomic DNA was used as template in a 25-μl PCR reaction mixture containing 200 μM dNTPs, 10 pmol of forward and reverse primers, and 1.5 U Taq DNA polymerase. As positive control, we used 50 pg of p35SGUSINT plasmid DNA. For PCR amplification, the samples were subjected to 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min followed by an extension cycle of 7 min at 72°C using a programmable Stratagene Robocycler Gradient 40. The PCR amplification products were resolved on a 1.4% agarose gel.

Southern blot analysis

Genomic DNA (10 μg) from 500 mg tissues was digested with *Hin*dIII for overnight at 37°C, electrophoresed on a 1.2% agarose gel, and transferred to a nylon membrane (Hybond-N; Amersham Biosciences, Little Chalfont, UK). The DNA was then cross-linked to the membrane by UV irradiation (1200 Joules in Stratalinker 100, Stratagene) and hybridized to a digoxigenin (DIG)-labeled DNA probe (Roche Diagnostics, Indianapolis, USA). The probe was produced by PCR-amplification of the entire *gus*-int sequence of the T-DNA region of p35SGUSINT (Fig. 1A) using the forward and reverse primers 5′-ATGTTACGTCCTGTAGAAACC-3′ and 5′-TCATTGTTTGCCTCCCTGCTG-3′, respectively (Bangalore, Genei, India). The hybridized probe was detected using alkaline phosphatase conjugate of an anti-DIG antibody and NBT/BCIP, a chromogenic substrate producing a blue-colored signal directly on the membrane at the site of the hybridized probe.

The *Agrobacterium* virulence (*vir*) gene induction assay

We used an octopine-type *Agrobacterium* strain A348 harboring the pSM219 plasmid that carries *lacZ* under the control of the *virH* promoter in *trans* to the wild-type pTiA6 plasmid (Stachel et al. 1985a; Stachel and Nester 1986). The reporter β-galactosidase activity was measured, and the results were expressed in specific units calculated as described (Miller 1972; Stachel et al. 1985a; Stachel and Nester 1986). Different concentrations of autoclaved L-glutamine were added to bacterial cultures in YMB, which were then grown overnight before measuring the β-galactosidase activity. Acetosyringone (AS, 100 μM) (Stachel et al. 1985b,

1986) was used as a positive control for maximal *vir* gene induction.

Quantification of bactericidal effects of tea leaf extracts

The average amount of polyphenols in tea leaves is 1 mg per 10 leaves (Kumar 2003). Based on this calculation, increasing amounts of extracts of fresh Kangra jat leaves, corresponding to 0.05, 0.1, and 1.0 mg/ml total polyphenols, were added to Luria Bertani (LB), YMB, or BM media solidified with 0.7% of either agar or agarose. Then, 200 μl of overnight EHA105 *Agrobacterium* cultures at cell density of 10^9 cells/ml were plated on each of these media and incubated at 28°C in dark for 24 h for agar-based media or for 48 h for agarose-based media. Following incubation, bacterial samples were harvested at 24-h intervals for 168 h, plated on YMB-agar medium, grown for 48 h at 28°C, and bacterial growth was quantified in colony forming units (cfu). The tested media contained L-glutamine (0.5, 1.0, and 2.0 g/l), or L-glutamine in combination with the indicated above amounts of leaf extracts. In control experiments, bacteria were grown on media without L-glutamine and leaf extracts.

Results

Agrobacterium-mediated transformation of tea leaves requires the presence of L-glutamine in the medium

Irrespective of pH or temperature, no *Agrobacterium*-mediated transformation, detected as transient expression of the *gus*-int reporter gene contained on the bacterial T-DNA, was observed in the leaves co-cultivated on any of the tested media, i.e., BM, SEM, and SRM without L-glutamine (Table 1). However, the GUS activity (Fig. 1B and C) was observed in leaves co-cultivated on each of these media supplemented with L-glutamine, but not in control, untransformed explants (Fig. 1D). Generally, the transformation occurred more efficiently at lower temperatures and acidic pH values, with the highest transformation efficiencies achieved at 19°C and pH 5.4 in all three media (Table 1). Because the *gus* reporter gene contained an intron which prevents its expression in bacteria (Vancanneyt et al. 1990), our GUS expression data represented the enzymatic activity directed by the T-DNA after its transfer to plant cells.

Transgene integration in the transformants

Integration of the reporter transgene into the leaf genome was demonstrated by PCR and Southern blot analyses. The PCR-based analysis of calli developed from leaves co-cultivated with *Agrobacterium* in the presence of L-glutamine showed the 600-bp PCR product corresponding to the predicted size of the amplification fragment based on the integrated *gus*-int gene (Fig. 2, lanes 1–4). No such amplification product was detected in untransformed leaf calli (Fig. 2, lane C).

Integration of the *gus*-int transgene into the genome of the isolated transformants was confirmed by Southern blot analysis of the transformed calli, in which the *gus*-int transgene was initially detected by PCR (see Fig. 2). We digested their genomic DNA with *Hin*dIII which is expected to excise the entire *gus*-int expression cassette from the integrated T-DNA (see Fig. 1A) and analyzed the resulting digests using the *gus*-int sequence as probe. Figure 3 shows that the transformed calli displayed the specific Southern hybridization signal corresponding to the expected size (ca, 2860 bp) of the *gus*-int expression cassette (lanes 1–3). As expected, no hybridization signal was observed with the untransformed calli (not shown) or with the calli derived from tissues which had been co-cultivated with *Agrobacterium*, but did not produce the *gus*-int-specific product in the PCR analysis (Fig. 3, lanes

Table 1 Transient expression of GUS activity contained within *Agrobacterium* T-DNA in response to L-glutamine at different temperatures, pH values, and co-cultivation media[a]

Medium	Temperature (°C)	Leaf explants expressing GUS (%)							
		L-glutamine (1.0 g/l)							
		pH 5.2		pH 5.4		pH 5.6		pH 5.8	
		+	−	+	−	+	−	+	−
BM	19	20	0	50	0	10	0	0	0
	22	10	0	30	0	10	0	0	0
	25	0	0	0	0	0	0	0	0
	28	0	0	0	0	0	0	0	0
SEM	19	30	0	60	0	0	0	0	0
	22	10	0	20	0	10	0	0	0
	25	0	0	0	0	0	0	0	0
	28	0	0	0	0	0	0	0	0
SRM	19	10	0	40	0	0	0	0	0
	22	10	0	30	0	0	0	10	0
	25	0	0	10	0	10	0	0	0
	28	0	0	0	0	0	0	0	0

[a]The data are derived from duplicate experiments each of which contained five replicates with ten leaves per tested condition.

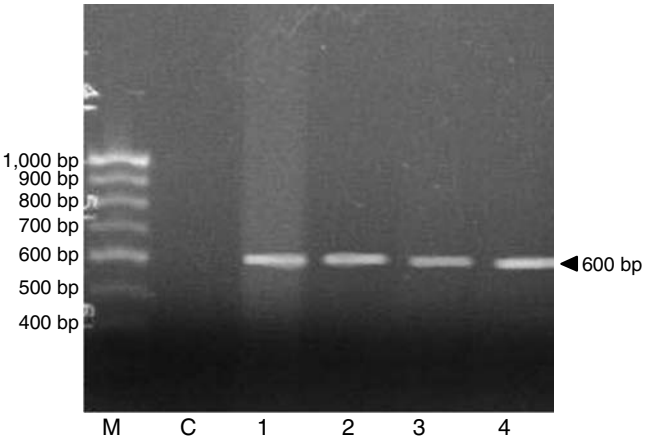

Fig. 2 PCR analysis of transgenic calli derived from tea leaf explants. Co-cultivation with *Agrobacterium* was performed on SEM at 19°C and pH 5.4. *Lane M*, molecular size markers; *lane C*, untransformed callus; *lanes 1–5*, independent transformants

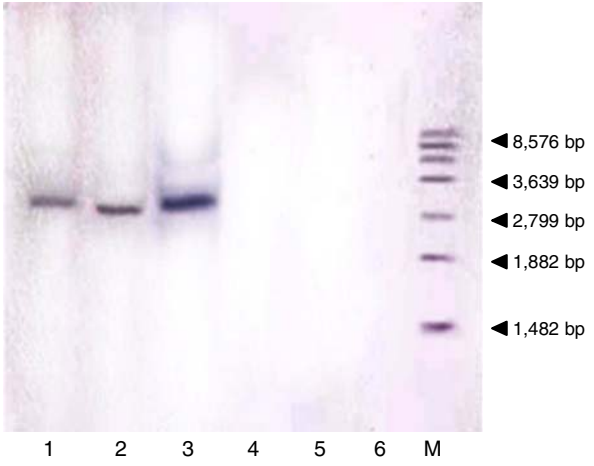

Fig. 3 Southern blot analysis of transgenic calli derived from transformed tea leaf explants. *Lanes 1–3*, independent transformants; *lanes 4–6*, calli from independent explants co-cultivated with *Agrobacterium*, but not transformed as determined by the PCR analysis; *lane M*, molecular size markers

4–6). Collectively, these results indicate that co-cultivation of tea leaf explants with *Agrobacterium* in the presence of L-glutamine results in stable genetic transformation.

Role of L-glutamine in gene transfer into plant tissues

The presence of L-glutamine in co-cultivation medium is absolutely required for *Agrobacterium*-mediated transformation of tea leaf explants. One possibility is that L-glutamine affects the induction of the bacterial *vir* genes which determine virulence. To test this idea, we used the *vir* gene induction assay, in which activation of *vir* promoters is determined from the enzymatic activity of the β-galactosidase reporter (Stachel et al. 1985a, 1986).

Table 2 shows that, in positive control experiments, the presence of AS, a known inducer of *vir* genes (Stachel et al. 1985b, 1986), resulted in high levels of the β-galactosidase activity. Addition of increasing concentrations of L-glutamine did not significantly alter the AS-induced *vir* gene expression levels. We observed a general "peak-shaped" trend of slight reduction in the *vir* gene expression at L-glutamine concentrations between 0.1 and 0.4 g/l, followed by a slight increase in the expression at concentrations of 0.5–0.6 g/l, and then another decrease at L-glutamine concentrations of 0.7–1.5 g/l (Fig. 4).

Table 2 L-Glutamine enhances *vir* gene induction by AS[a]

Compound tested	β-Galactosidase activity (units)
No addition	66.52 ± 1.12[b]
AS[c]	147.22 ± 1.09
AS + L-glutamine (0.1 g/l)	114.10 ± 1.94
AS + L-glutamine (0.2 g/l)	125.23 ± 1.23
AS + L-glutamine (0.4 g/l)	129.31 ± 1.08
AS + L-glutamine (0.5 g/l)	194.27 ± 0.81
AS + L-glutamine (0.6 g/l)	156.86 ± 1.49
AS + L-glutamine (0.7 g/l)	144.16 ± 1.30
AS + L-glutamine (1.0 g/l)	131.31 ± 1.48
AS + L-glutamine (1.5 g/l)	131.47 ± 1.60

[a]The data are derived from duplicate experiments each of which contained five replicates with 10 leaves per tested condition.

[b] $\pm$ Standard deviation.

[c]AS concentration is 100 μM.

Next, we examined the effect of tea leaf extracts and L-glutamine on bacterial growth on different media. Three specific parameters were tested: the amount of L-glutamine, the amount of tea leaf extract (i.e., native *vir* gene inducer), and the solidifying agent of the co-cultivation medium (i.e.,

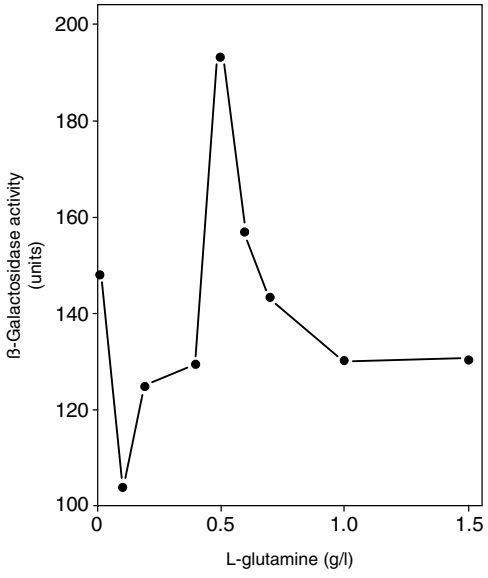

Fig. 4 AS-induced *vir* gene expression as function of L-glutamine concentration in the co-cultivation medium. The curve was constructed based on the quantitative data presented in Table 2

Table 3 *Agrobacterium* growth in the presence of tea leaf extracts and L-glutamine[a]

BM medium supplemented with	Number and size[b] of colonies					
	Agar			Agarose		
	24 h	48 h	168 h	24 h	48 h	168 h
None	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
Leaf extract (0.05 mg/ml)	None	>500, small	Confluent growth	None	>500, small	Confluent growth
Leaf extract (0.1 mg/ml)	None	18	24	None	9	38
Leaf extract (1.0 mg/ml)	None	4	28	None	2	29
L-Glutamine (0.5 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
L-Glutamine (1.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
L-Glutamine (2.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
Leaf extract (0.05 mg/ml) L-Glutamine (0.5 g/l)	8	26	>500, small	10	63	–
Leaf extract (0.1 mg/ml) L-Glutamine (0.5 g/l)	None	82	119	None	86	330
Leaf extract (1.0 mg/ml) L-Glutamine (0.5 g/l)	None	6	211	None	None	None
Leaf extract (0.05 mg/ml) L-Glutamine (1.0 g/l)	>500, small	>500, small	Confluent growth	58	>500, small	Confluent growth
Leaf extract (0.1 mg/ml) L-Glutamine (1.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
Leaf extract (1.0 mg/ml) L-Glutamine (1.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
Leaf extract (0.05 mg/ml) L-Glutamine (2.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
Leaf extract (0.1 mg/ml) L-Glutamine (2.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth
Leaf extract (1.0 mg/ml) L-Glutamine (2.0 g/l)	>500, small	>500, large	Confluent growth	>500, small	>500, large	Confluent growth

[a]The data are derived from duplicate experiments each of which contained five replicates with 10 leaves per tested condition.

[b]Colonies 0.4–0.8 mm in diameter were considered small, and colonies with a diameter of $\geq$ 1.0 mm were considered large.

agar or agarose). Table 3 shows that while very low concentration of the leaf extract (0.05 mg/ml) allowed growth of numerous large colonies after 48 h, as few as 4–18 cfu were observed at higher leaf extract concentrations (0.1 and 1.0 mg/ml) after 48 h, and this low level of bacterial growth did not increase beyond 24–38 cfu even after 168 h.

In the absence of leaf extracts, numerous colonies were observed on both agar- as well as agarose-solidified media supplemented with L-glutamine at all tested concentrations (0.5, 1.0, and 2.0 g/l); this growth was essentially identical to that observed on the control media, devoid of L-glutamine (Table 3). These results indicate that the tested concentrations of L-glutamine had no detectible effect on *Agrobacterium* growth.

When L-glutamine was used in combination with leaf extract in the co-cultivation medium, higher concentrations of L-glutamine (1.0 and 2.0 g/l) were effective in promoting bacterial growth even at 0.1 and 1.0 mg/ml of tea leaf extract after 24 h. Under conditions where bacterial growth was observed after 24 h, the colonies were numerous and small, and they increased in size after 48 h of growth (Table 3).

The nutritional composition of different basic media (i.e., MSO, YMB, and LB) had no significant effect on the bacterial growth (not shown). The nature of the solidifying agent (i.e., agar or agarose) also did not affect the growth of *Agrobacterium* (Table 3). Furthermore, when we tested other amino acids, e.g., aspartic acid, glutamic acid, arginine, phenylalanine, valine, asparagine, threonine, lysine, and serine, none of them—even L-asparagine which has a chemical composition similar to that of L-glutamine—were able to allow growth (not shown).

Discussion

Successful genetic transformation of tea leaf explants requires that three general conditions are met: (i) the bactericidal effect of leaf polyphenols has to be overcome, (ii) *Agrobacterium vir* gene expression must occur, and (iii) the transformed leaf explants must retain their potential for shoot regeneration. To date, the commonly used antioxidants or polyphenol adsorbents have not only been unable to negate the bactericidal effect of leaf polyphenols, but also

inhibited the regeneration process (Kumar 2003). Here, we discovered that L-glutamine protects *Agrobacterium* from the bactericidity of tea leaf explants and preserves the bacterial virulence. Indeed, the addition of L-glutamine to different co-cultivation media substantially facilitated *Agrobacterium* infection of the leaf explants in the cultivar Kangra jat of tea and resulted in the T-DNA transfer and integration into the tea genome. This finding is important because *Agrobacterium*-mediated genetic transformation of leaf explants of this and other important tea cultivars has been difficult (Biao et al. 1998). On the other hand, it is the leaves that, unlike cotyledons and embryonic axes, have the potential to retain the clonal fidelity of the superior tea plants (Sandal et al. 2005) of the Chinary type cultivar Kangra jat which yields a high value Kangra tea that comes second only to Darjeeling tea (Bhattacharya et al. 2005). Thus, as compared to other explants that have been commonly used in tea transformation (Mondal et al. 2002; Lopez et al. 2004), it is more desirable to use leaves for producing transgenic plants as important genes can be stacked onto the already existing superior traits of a selected clone (Sandal et al. 2005). Moreover, the loss of these desired traits through genetic segregation in transgenic plants produced from leaf explants is eliminated as tea is mainly a clonally propagated crop plant (Barua 1989).

We obtained two important insights into the mechanism by which L-glutamine facilitates the *Agrobacterium*-mediated gene transfer into tea leaves. First, L-glutamine, unlike the commonly used polyphenol adsorbents and antioxidants, preserves the ability of *Agrobacterium* to employ its virulence system, i.e., *vir* genes, for T-DNA transfer and does not have adverse effects on the plant regeneration process. Second, L-glutamine counteracts the bactericidal effects of polyphenols that are exuded from tea leaves when used as explants (Sandal et al. 2004; Hamilton-Miller 1995). Indeed, xenobiotic detoxification is not uncommon in different systems including bacteria (Tschech and Fuchs 1987; Coleman 1997; Rousseaux et al. 2001), and glutarimides, that are generally formed on thermal degradation of glutamine, are known to have xenobiotic properties (Kalgutkar et al. 2002). Consistent with this idea, L-glutamine sustained vigorous *Agrobacterium* growth on media containing high concentrations of tea leaf extracts which, in the absence of L-glutamine, completely blocked bacterial colony formation. These findings help to better understand the recalcitrance of tea leaf tissues to *Agrobacterium* infection and develop a simple experimental protocol for overcoming this difficulty in genetic manipulation of commercial tea cultivars.

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