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Genetic transformation of a local variety of tomato using *gus* gene: an efficient genetic transformation protocol for tomato

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An efficient and reproducible transformation protocol was optimized for L15, a local tomato variety using *Agrobacterium* strain GV 2260 carrying β-*glucoronidase* gene on pCAMBIA 1301 plasmid with *hpt*I gene as a plant selection marker. The use of pre-cultured explants (cotyledon leaf and hypocotyl) helped not only in easy handling of explants during transformation experiments, but also in reducing the *Agrobacterium* contamination and death before callusing due to insufficient strength of explants to bear the antibiotic treatments. *Agrobacterium* cell density (OD_{600}) and immersion time in bacterial solution proved detrimental for the survival of the explants during post cocultivation steps. Cocultivation time of 48 hours and a cefotaxime concentration of 300 mg l^{-1} were found to be ideal to keep the *Agrobacterium* under control during the transformation experiments. Inspite of taking sufficient care during cocultivation and post cocultivation steps, 2.83 % of explants responded with good quality callus out of two forty seven explants infected rest of the explants turned black and dried on Selection Medium. Although callus *gus* assay proved positive for all the samples only few (2) islets of the callus samples were transformed completely and the rest showed the expression of the *gus* gene as specks of *Agrobacterium* infection on the callus clump. The callus samples were selected for second round on callus regeneration medium could give few islets (3-4) of regenerating plantlets on each clump. The plantlets thus obtained were elongated, rooted and were transferred into pots in the glass house. The *gus* assay of the leaf samples from the plantlets obtained gave completely transformed plants expressing the *gus* gene in the entire leaf.

Key words: *Agrobacterium* mediated transformation, Immersion timing, *Agrobacterium* cell density, cocultivation time, *Gus* gene, reporter gene, Gus histochemical assay

Introduction

Tomato is one of the most important vegetable crops throughout the world and is available in almost all seasons of the year in tropical and sub-tropical

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regions. It is highly nutritive and consumed both directly and by processing. This has served as model plant for cloning agronomically important genes in dicotyledonous crop plants (Wing *et al.,* 1994). It has a relatively small genome (0.7-1.0 pg), well developed classical (Rick and Yoder, 1988) and molecular genetic maps (Tansksley *et al*., 1992) and a complete genomic library in yeast artificial chromosomes. The first resistant gene (*Pto*) that elicits a hypersensitive response to disease resistance was cloned in tomato (Martin *et al*., 1993). The natural ability of *Agrobacterium tumefaciens* in infecting only dicotyledonous plants because of the signalling acetosyringone phenolic compound released from the wounds of the plant cells has paved the way for the researchers in attaining more understanding and a precise manner of working upon this process. However, other methods of creating genetic manipulation are also in use such as particle gene gun technology, electroporation, protoplast mediated, polyethylene glycol mediated transfer, microinjection (Riva *et al.,* 1998) etc. *Agrobacterium* mediated transformation still has remarkable advantages over other transformation methods which includes preferential integration of defined T-DNA into transcriptionally active regions of the chromosome (Czernilofsky *et al*., 1986; Le *et al*., 2001; Olhoft *et al*., 2004) with exclusion of vector DNA (Hiei *et al*., 1997; Fang *et al*., 2002), unlinked integration of co-transformed T-DNA (McKnight *et al*., 1987; Komari *et al*., 1996; Hamilton, 1997; Olhoft *et al*., 2004). The transgenic plants are generally fertile and the foreign genes are often transmitted to progeny in a Mendelian manner (Rhodora and Thomas, 1996).

The transfer of T-DNA and its integration into the plant genome is influenced by several factors such as plant genotype, selection of the explant, vectors-plasmid designed, bacterial strain, addition of vir-gene inducing synthetic phenolics compounds, composition of culture media, tissue damage, suppression and elimination of *A. tumefaciens* infection after cocultivation (Alt-morbe *et al*., 1989; Bidney *et al*., 1992; Hoekema *et al*., 1993; Hiei *et al*., 1997; Komari *et al*., 1996; Nauerby *et al*., 1997; Klee, 2000). The first successful *Agrobacterium* mediated transformation of tomato was done in 1986 (McCormick *et al*., 1986). Although transformation of tomato has been reported time and again, it is still far from routine methods. Many unknown factors determine the rate of success, depending on cultivar, *Agrobacterium* strain and antibiotic selection system. Understanding the *in vitro* behavior of different genotypes of tomato and working out an efficient transformation protocol in a given set of genotypes is necessary to harness the benefit of candidate genes for pest and disease resistance. Thus, an attempt was done to introduce the pod borer resistance gene (Insecticidal cry protein gene {ICP}) into the local genotype available after the optimization of the efficient tissue culture protocol (In press). Before entering into the exact process of transformation a brief optimization for the genetic transformation was tried using

gus gene construct. Here we present the optimization of the transformation protocol for the local variety Megha (L15) using *gus* reporter gene considering different parameters viz., pre-cultivation of explants, immersion timing of the explants in *Agrobacterium* suspension, *Agrobacterium* cell density, cocultivation timing and the concentration of cefotaxime.

Materials and methods

Explant

Seeds of megha (L15) were obtained from Department of Horticulture, University of Agricultural Sciences (UAS), Dharwad. The seeds were bleached with 1% Sodium hypochlorite solution for 10 min, then thoroughly washed with running tap water, followed by surface sterilization with 0.1% (w/v) aqueous mercuric chloride solution for 3 minutes inside laminar hood and finally washed five times with sterilized distilled water and blot dried on sterile filter paper. Twenty five seeds per bottle were aseptically sown in jam bottles containing 50 ml of seed germination medium and incubated in the dark for 1 week. The bottles after a week were transferred to the light to avoid lanky seedlings. The plant tissue culture tubes with 15ml of the culture medium were used in all the *in vitro* experiments only exception for pre-culturing of explants on petri plates. Cotyledonary leaf and hypocotyl explants were excised from 15-day-old seedlings and were used for *Agrobacterium* cocultivation after 48 hrs of pre-culturing. Seedlings and all *in vitro* plant materials were incubated at 25±2° C under a 16/8-h (day/night) photoperiod. Light was provided by cool-white fluorescent lamps at photosynthetic photon flux of 60 µE m^{-2} s^{-1}. The *in vitro* culture experiments were conducted at the tissue culture laboratory, Department of Biotechnology/ Department of Horticulture, College of Agriculture, University of Agricultural Sciences (UAS), Dharwad.

Culture media

In all culture media, MS medium (Murashige and Skoog, 1962) mineral salts supplemented with 0.8 % agar and 3 % sucrose was used as the basal medium. For the aseptic germination of seeds, 1/2-strength MS basal medium was used. The induction of transgenic callus was achieved on MS medium supplemented with 2 mg l^{-1} 2, 4-Diaminetetraacetic acid (2,4-D) and differentiation of shoot buds on medium containing 3 mg l^{-1} kinetin + 0.3 mg l^{-1} IAA with hygromycin (3 mg l^{-1} for callus and 5 mg l^{-1} for plantlets) as selective agent. The antibiotic cefotaxime (300 mg l^{-1}) was used to overcome *Agrobacterium* contamination in the selection medium. For normal shoot development, shoot buds were cultured on MS basal

medium containing 5 mg l^{-1} kinetin + 5 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime for 15 days. Shoots were rooted on MS medium containing 0.3 mg l^{-1} IAA and 3 mg l^{-1} hygromycin.

Bacterial strain and vector

Agrobacterium tumefaciens strain GV 2260 (Vanacanny *et al*., 1990) harbouring the plasmid pCAMBIA 1301 with the β-glucuronidase (GUS) gene interrupted with a plant intron (GUS-INT) (Vancanney *et al*., 1990) driven by the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) and nopaline synthase terminator as reporter gene was used for transformation (Fig. 1). This vector has hygromycin phosphotransferase (hptI) gene in T- DNA region driven by CaMV35S promoter and CaMV35S polyA terminator confers resistance to the antibiotic hygromycin as a plant selection marker and neomycin phosphotransferase (nptII) gene driven by the CaMV35S promoter and nopaline synthase (NOS) terminator outside to T-DNA region, which confers resistance to the antibiotic kanamycin as a bacterial selection marker.

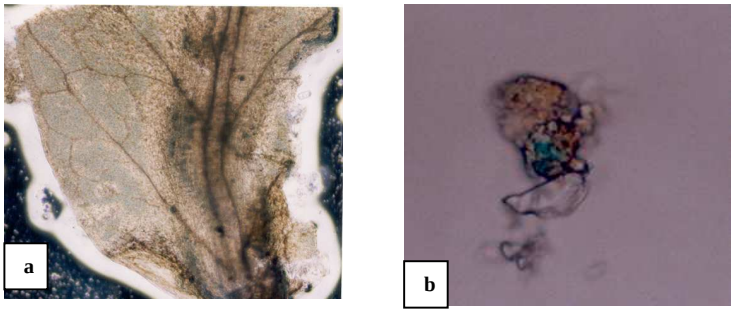

Fig. 1. Expression of GUS gene among putative transgenics in leaf a: and callus b: samples

Transformation procedure and plantlet formation

For transformation experiments a single Agrobacterium colony from freshly subcultured plate was grown overnight in YEM liquid medium with the appropriate antibiotics (50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin) at 28°C in a rotary shaker (125 rpm). Explants from 15-day-old seedlings of the variety megha were precultured on the callus induction medium (2 mg l-1 2, 4-Diaminetetraacetic acid (2, 4-D) for 48 hours on petri plates. For cocultivation, the pre-cultured explants were incubated for various time intervals for 0, 3, 5, 7, 10, 15 and 20 minutes in the *Agrobacterium* culture with OD_{600} of 0 (YEM broth), 0.1, 0.3, 0.5, 0.7 and 1 to find out the ideal immersion timing and the optimum *Agrobacterium* cell density for the survival of explants during post cocultivation experiments. The explants immersed

in the *Agrobacterium* suspension were then transferred onto the callus induction medium tubes and allowed for the cocultivation period of 0, 24, 48, 72 and 96 hours to know the ideal time period required for the transfer of T-DNA. After cocultivation period the explants were washed repeatedly using sterile distilled water with 500 mg l^{-1} cefotaxime to get rid of the *Agrobacterium* contamination and were placed on selection medium with different concentrations of cefotaxime viz., 0, 100, 200, 300, 400 and 500 mg l^{-1} to find out the suitable concentration of cefotaxime to avoid the bacterial contamination during callus formation and morphogenesis stages. Elongated shootlets were rooted in MS medium containing 0.3 mg l^{-1} IAA along with 3 mg l^{-1} hygromycin. Culture tubes containing rooted shoots were kept open inside the culture room for 2 days for hardening. The next day rooted shoots were washed to remove the agar and immersed completely in tap water for 5 minutes under direct sunlight and were directly transferred onto pots containing a mixture of 2:1:1 of soil: sand: peat and maintained in the glasshouse. Observations were recorded on the survival of explants periodically from each experiment tried respectively.

Identification of transgenic callus and plants by histochemical assay

The putative transformants (both callus and leaf samples) were histochemically detected for the presence of the *GUS* gene following the procedure of Jefferson *et al.* (1987). The samples from the putative callus and transgenic plants were incubated in a solution containing the stocks of 2 mM X-glucuronide in DMSO, 100mM Tris HCl (pH 7.0), 50 mM NaCl, 2 mM potassium ferricyanide and 0.1 % (v/v) triton X-100 pipetted in required quantities to make *gus* staining solution overnight at 37°C and cleared in 75% ethanol for 4 hours to remove the chlorophyll and clean the tissue. The tissues or calli were inoculated on a glass slide and observed under microscope (Olympus) cells and tissues for blue staining.

Results and discussion

Over the last two decades a whole lot of improvement in the plant transformation process has taken place giving rise to several modifications in the protocols followed by different researchers and for different causes or purposes. These modifications are obvious in this area of research, since variations are found in each and every step of the transformation process followed viz., the genotype, explant source and characterstics, culture media type and conditions, different growth regulators use and concentrations, bacterial strains and gene constructs deployed, their virulence levels, type of selectable marker etc. Thus, there are several methods used for transformation of plants like particle bombardment, electroporation, protoplast mediated, pollen mediated, *Agrobacterium* mediated

approach, direct DNA transfer methods very recently. *Agrobacterium* mediated approach remains the method of choice because of its understanding over the process and the more clear explanation over this procedure. This approach has more advantages than the other methods followed (Czernilofsky *et al*., 1986; Le *et al*., 2001; Olhoft *et al*., 2004; Hiei *et al*.; 1997; Fang *et al*., 2002). The step by step explanation of this method ensures the reliable transfer of T-DNA into the plant genome and can be backcrossed into desired genotype. Single copy transfer of T-DNA is possible with this method compared to other methods where direct DNA transfer is followed and very difficult to get single copy number.

The competency of the plant cells to T-DNA delivery can be obtained by manipulation of the explant *in vitro*. Pre-culturing the explants for 48 hours before cocultivation gave very good response compared to that of without pre-culturing before cocultivation. Thus, pre-culturing explants served better in assisting the easy handling of the explants, proper drying and avoiding the explant death during post cocultivation steps, because of their increased size after pre-culture for both the explants. Transgenic groundnut (*Arachis hypogaea* L.) plants were produced efficiently by inoculating different explants with *Agrobacterium tumefaciens* strain LBA4404 harbouring a binary vector pBM21 containing *uidA* (*GUS*) and *nptll* (neomycin phosphotransferase) genes. Genetic transformation frequency was found to be high with cotyledonary node explants followed by 4 days cocultivation and this method required 3 days of precultivation period before cocultivation with *Agrobacterium* (Venkatachalam *et al.,* 1998). Precultivation of apple explants before cocultivation has increased the number of *gus* expressing zones measured immediately after cocultivation, but drastic reduction in the number of *gus* expressing transformed calli found on the explants 6 weeks after infection (An De Bondt *et al.,* 1994). Four days of pre-culture and 2 days of co-cultivation were optimum for *Melastoma malabathricum* transformation, while 3 days of pre-culture and co-cultivation for *Tibouchina semidecandra* as reported by Wilson Thau Lym Yong *et al.* (2006). The optimized transformation protocol for *Lycium barbarum* included pre-culture of leaf explants for 3 days on the medium for callus induction followed by inoculation with *Agrobacterium* strain EHA101 (pIG121Hm), cocultivation for 3 d at 24°C and using this protocol, 65% L. barbarum explants gave rise to Kan-resistant and GUS-positive calli (Zhong Hu *et al*., 2006).

The Agrobacterium cell density used for the cocultivation is equally important factor for the transformation experiments as the explant used for cocultivation differs in the strength for overcoming the effect of immersion in *Agrobacterium* culture containing medium. So, out of the five concentrations tried an OD_{600} of 0.3 was suitable for the survival of the explant after immersion in the bacteria containing medium for 2 minutes. Most of the experiments uses above 0.5 OD_{600} for cocultivation, but since the explants were tiny and very tender and the

mortality of the explants was found more even at 0.5 OD_{600} a very low OD_{600} of 0.3 where the *Agrobacterium* cell count is minimum was used (Table 1). A cocultivation incubation period of 48 hours was suitable for the explant to survive from the *Agrobacterium* contamination after incubation and help the proper transfer of T-DNA. In *Melastoma malabathricum* and *Tibouchina semidecandra*, a concentration of 1×10^7 cfu ml^{-1} (OD_{600} 0.8) has shown the highest virulence *in* the *Agrobacterium tumefaciens* strains LBA4404 and EHA105 respectively (Wilson Thau Lym Yong *et al.,* 2006). Leaf explants from 'Gala' and 'McIntosh Wijcik' apples where explants are sturdy enough responded with the highest regeneration rate and high T-DNA transfer efficiency when using *Agrobacterium* cells of OD_{600} 0.9 to 1.1 incubated at 28° for 24 hours in liquid YEB medium (Song *et al.,* 2001). This indicates that the strength of the explant used for infection influences most on the *Agrobacerium* cell density concentration (OD_{600}). Influence of cocultivation time on transformation efficiency of *Arabidopsis* cell cultures by *Agrobacterium* gave the increased number of transformed calli with the increase in the cocultivation time of Arabidopsis cells with *Agrobacteria*, reaching an optimum after 48 hours. Longer cocultivation time led to the reduced viability of the plant cells and thus to a delay of the growth of transformed microcalli (Christoph *et al.,* 1997).

Further during the subculturing stages of callus, the explants were able to rescue from the *Agrobacterium* contamination by culturing the infected explants on the subculturing medium supplemented with different concentrations of cefotaxime. Hypocotyl explants showed survival on 300 mg l^{-1} cefotaxime containing media till seven days, whereas Cotyedonary leaf explants were able to survive on the same medium for only five days. At 300 mg l^{-1} of cefotaxime in the media the *Agrobacterium* contamination was successfully overcome during the subculturing (Table 2). Here out of 247 explants inoculated on the hygromycin containing callus induction medium only 7 explants were able to overcome the selection pressure and induce the callus, while the rest of the explants turned black and died (Fig. 1). This critical selection pressure helped in easing the optimization of the protocol for genetic transformation of the *gus* gene introduction into the megha variety of tomato. The increase in number of escapes, the regeneration of non transformed plants can be explained by an inefficient selection due to the protection of non transformed cells from the selection agent by surrounding transformed cells (Ghorbel *et al.,* 1999). After 4 weeks of culture, calli developing from the explants were separated into two clumps, one for *gus* assay and other for subculturing into shoot-bud initiation through second round selection. Shoot-bud elongation was tested on basal medium containing 5 mg l^{-1} hygromycin and 300 mg l^{-1} cefotaxime. Elongated shootlets were rooted in MS medium containing 0.3 mg l^{-1} IAA along with 3 mg l^{-1} hygromycin. Rooted shoots containing culture tubes were kept open in culture room for 2 days for hardening. The next day rooted

shoots were washed and immersed completely in tap water for 5 minutes under direct sunlight and were directly transferred to pots containing a mixture of 2:1:1 of soil: sand: peat and maintained in the glasshouse.

Table 1. Effect of concentration (OD600) of bacterial suspensionon survivability of explants upon cocultivation for 2 minutes.

O D after diln	Survival of explants after incubation (Hours)							
	24		48		72		96	
	CL	Hy	CL	Hy	CL	Hy	CL	Hy
0	H	H	H	H	H	H	H	H
0.1	H	H	H	H	H	H	Y	Y
0.3	H	H	H	H	Y	Y	D	D
0.5	H	H	H	H	Y	H	D	D
0.7	H	H	D	Y	D	D	D	D
1.0	H	H	D	D	D	D	D	D

Note: CL: Cotyledonary leaf Hy: Hypocotyl H: Healthy Y: Yellowing D: Dead

Callus samples of all the seven explants obtained were found to express the *gus* gene under *gus* assay indicating the complete transformation of the explants infected and the efficiency or criticality of the selection process using hygromycin. The callus subjected to regeneration infact yielded all transformed plants with complete *gus* gene expression in the whole leaf blade (Fig. 2). *GUS* assay on regenerated plants indicated the expression of *uidA* gene in all of them. With this experiment we have optimized parameters during transformation process viz., pre-cultivation of explant, *Agrobacterium* cell density (OD_{600}), Immersion timing of explant with *Agrobacterium* suspension, cocultivation period and cefotaxime concentration and this method has been efficiently utilized for developing *Cry1Ab* gene carrying tomato plants with good expression levels of mRNA and CRY protein as confirmed by insect bio-assay data (unpublished). Thus, we report an efficient genetic transformation protocol with *Agrobacterium* mediated approach in Megha (L15) genotype of tomato.

Table 2. Effect of levels of cefotaxime on survival of explants after different time intervals.

Cefotaxime (mg l-1)	Survival of explants after inoculation (days)									
	3		5		7		9		11	
	CL	Hy	CL	Hy	CL	Hy	CL	Hy	CL	Hy
0	Y	Y	D	D	D	D	D	D	D	D
100	H	H	Y	Y	D	D	D	D	D	D
200	H	H	Y	Y	D	D	D	D	D	D
300	H	H	H	H	D	H	D	D	D	D
400	H	H	H	H	D	D	D	D	D	D
500	H	H	H	H	D	D	D	D	D	D

Note: CL: Cotyledonary leaf Hy: Hypocotyl H: Healthy Y: Yellowing
D: Dead

Fig. 2. Regeneration from callus on hygromycin selection medium.

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