



## RESEARCH PAPER

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## Establishment and optimization of *Agrobacterium*-mediated transformation and regeneration of tomato (*Solanum lycopersicum* L.)

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### Abstract

An efficient procedure for *Agrobacterium*-mediated transformation of tomato using in vitro derived cotyledon and leaf-disc explants for the recovery of non-chimeric stable transgenic plants has been developed. Several parameters including pre-incubation of explants, *Agrobacterium* cell density, co-cultivation conditions, selection regime and media fortification, have been extensively optimized. Fortification of 3 % maltose in shoot induction medium (SIM), 3 % sucrose in shoot elongation medium (SEM) and 2 % sucrose in root induction medium (RIM) showed best results using in vitro leaf-discs (2–5 mm), whereas 2 % sucrose was found optimum using cotyledon (8 mm) explants from in vitro germinated seeds. Leaf explants showed 96.2 % response with 5.8 mean numbers of elongated shoots per explant in tomato cultivar PED. Optimal hormone additives were 2.5 mg l<sup>-1</sup> 6-benzyladenine (BA) + 0.5 mg l<sup>-1</sup> indole-3-acetic acid (IAA) in SIM, and 1.0 mg l<sup>-1</sup> gibberellic acid (GA<sub>3</sub>) in SEM for leaf discs; versus 1.0 mg l<sup>-1</sup> zeatin (ZET) and 0.2 mg l<sup>-1</sup> IAA for cotyledon explants. Half-strength MS medium with 0.5 mg l<sup>-1</sup> indole-3-butyric acid (IBA) in RIM was optimum for rooting, from both explant sources. The two-step selection cycle resulted in transformation efficiency from 3.17 to 21.38 % for cotyledon explants and 21.83 to 35.70 % for leaf explants in tomato cultivar PED using selection based on either *nptII*, *hptII* or *bar* genes. The optimized conditions for co-cultivation, *in vitro* screening and development of transgenic plants were suitable for the transfer of genes of diverse origin into Indian cultivars of tomato.

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## Introduction

*Agrobacterium*-mediated genetic transformation of plants has become an integral tool in plant biotechnology for both basic and applied research including functional genomics. Transgenic technology has tremendous potential to address various biological issues related to crop improvement, including higher yield, tolerance to abiotic and biotic stress, and expression of desired traits of diverse origin which are otherwise difficult to achieve by conventional breeding approaches (Liénard *et al.*, 2007; James, 2010; Park *et al.*, 2011). Successful DNA-mediated plant transformation requires a simple, reproducible, genotype-independent and cost-effective regeneration protocol, competent for genetic transformation, regeneration and recovery of fertile stable transgenic events. In addition, the protocol should avoid introducing somaclonal variation and creation of chimeric plants, especially due to the long duration of tissue culture steps (Birch, 1997; Gelvin, 2003; Bergounoux, 2014). Several recalcitrant important crop plants like cotton, soybean, maize, rice, canola, groundnut and pea have been transformed using different strains of *A. tumefaciens* in the last two decades for development of transgenic plants with desired traits. The success rate in developing transgenic plants have been low due to several physiological and biochemical conditions of genetic transformation (Tzfira and Citovsky, 2000; Zupan *et al.*, 2000).

Tomato (*Solanum lycopersicum* L.) is an economically important vegetable crop and is grown worldwide over 4.8 million hectares, for fresh market and food processing industries (FAOSTAT, 2012). It is a rich reservoir of important nutrients such as lycopene,  $\beta$ -carotene and vitamin C. Therefore, introduction of qualitative genetic traits in commercial cultivars of tomato is essential to improve its nutritional quality, productivity, tolerance to biotic and abiotic stresses, and deployment in biopharming (Bergounoux, 2014). The availability of the tomato genome sequence and a large number of SNPs will guide breeders in identification of the important genes for biodiversity-based breeding (Sato *et al.*,

2012). The introgression of agronomically important traits in commercial varieties of tomato by direct DNA-mediated transformation through transgenic technology is a promising alternative. McCormick *et al.*, (1986) had first successfully documented tomato transformation and subsequently several reports on genetic transformation in different species and cultivars of tomato using different explants and culture conditions have been demonstrated (Frary and Earle, 1996; Velcheva *et al.*, 2005; Kaur and Bansal, 2010; Thi Van *et al.*, 2011; Rai *et al.*, 2012; Khuong *et al.*, 2013).

The primary mode of regeneration in tomato is *via* shoot organogenesis from dedifferentiating callus derived from various explants namely leaf, hypocotyl, cotyledon or directly from a thin layer of cells from pedicel and peduncle, depending upon the genotype (Bhatia *et al.*, 2004). The factors which greatly influence *Agrobacterium*-mediated genetic transformation include the nature and age of the plant material, several physiological and biochemical conditions of *Agrobacterium* infection, and the selection regime (Frary and Earle, 1996; Park *et al.*, 2003; Gao *et al.*, 2009; Li *et al.*, 2013). For some plants, *Agrobacterium*-mediated transformation followed by regeneration *via* direct organogenesis requires managing the high-frequency of escapes, chimeric nature of regenerated transgenic plantlets, inconsistency, and low transformation frequencies owing to complexities of the *in vitro* regeneration and non-rigorous selection procedures (Mathews *et al.*, 1998; Duclercq *et al.*, 2011). Establishing a routine, robust, genotype-independent, and high frequency transformation system in tomato is essential for its functional genomics and genetic improvement (Sun *et al.*, 2006).

Several modifications and strategies like application of a liquid culture system, feeder layer and high selection pressure of antibiotics during shoot and root development have been documented to cope with the problem of chimeras and escapes for recovery of true transgenic plants (Velcheva *et al.*, 2005; Sun *et al.*, 2006). In the present study, we have optimized high

frequency regeneration of excised cotyledon and leaf explants in six Indian commercial cultivars of tomato following *Agrobacterium* co-cultivation, leading to the recovery of non-chimeric and high frequency of stable transgenic plants. The results obtained with different genes and selection markers advocate the effectiveness, efficiency and simplicity of the procedure for recovery of stable transgenic plants.

## Materials and methods

### *Bacterial strain and plasmids*

*A. tumefaciens* strain LBA 4404 harbouring the 14.8 kb vector p35SGUS-INT with the *uidA* gene for  $\beta$ -glucuronidase activity and *nptII* gene for kanamycin resistance was used for the optimization of different parameters crucial for *Agrobacterium*-mediated transformation of tomato (Vancanneyt *et al.*, 1990). The T-DNA regions of different plant vectors pBIN200 (*Bt-cry1Ab*), pPAK ( $\alpha$ -*PI*), pBIN442 (*taf4b*), pBIN1612 (*nbri12*) and pBIN1613 (*gfp-nbri12*) used for validation of the tomato transformation procedure are shown in Fig. 1. For agro-inoculation, bacterial cultures of *A. tumefaciens* harbouring different plant vectors were grown overnight at 28 °C in YEB medium containing 20 mg l<sup>-1</sup> rifampicin, 50 mg l<sup>-1</sup> kanamycin and 50 mg l<sup>-1</sup> streptomycin.

### *Plant material and growth conditions*

Seeds of tomato (*Solanum lycopersicum* L.) cultivar Pusa early dwarf (PED) were used in pilot studies to optimize the transformation and regeneration protocol. Additional cultivars were analyzed using the conditions determined to be optimum for PED; these cultivars were Pusa 120, Pusa hybrid 1, S-22, Pusa Ruby and Gaurav and were obtained from National Seeds Corporation, India.

The seeds were washed with 0.1 % (v/v) labolene detergent (Qualigens, Mumbai, India) followed by 3 rinses with sterile distilled water, subsequently, treated with 70 % (v/v) ethanol solution for 90 s and rinsed 4–5 times with sterile distilled water. The seeds were then treated with 4 % (v/v) sodium hypochlorite solution for 15 min, rinsed 5–6 times

with sterile water, dried and seeded onto semi-solid MS medium (Murashige and Skoog, 1962) containing B5 vitamins instead of MS vitamins, 3 % (w/v) sucrose (HiMedia Labs, Mumbai, India) and 0.8 % (w/v) agar (Sigma, USA) and incubated at 24 ± 2°C in a culture room at 16 h photoperiod with photon flux intensity of 80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The *in vitro* germinated cotyledons were excised from 10-day-old seedlings while leaf-discs were prepared from 16–18 days old *in vitro* grown plantlets. The cotyledon and leaf-disc explant preparation is shown in Fig 2A.

### *Co-cultivation and selection of transformed plantlets*

Excised cotyledons were initially pre-incubated on semi-solid MS medium supplemented with 2 % (w/v) sucrose + 1.0 mg l<sup>-1</sup> ZET + 0.2 mg l<sup>-1</sup> IAA and leaf discs on 3 % (w/v) maltose + 2.5 mg l<sup>-1</sup> BA + 0.5 mg l<sup>-1</sup> IAA, for three days prior to agro-inoculation (Fig 2A). Excised cotyledon explants were treated with *Agrobacterium* suspension in liquid co-cultivation medium (pH 5.6) for 20 min at OD<sub>600</sub> between 0.2–0.25 with 2 × 10<sup>9</sup> cells ml<sup>-1</sup> measured by means of a haemocytometer (Sigma, USA). The cotyledon explants were then removed, dried on sterilized blotting paper and transferred onto co-cultivation medium comprising of MS salts (with B5 vitamins) + 2 % (w/v) sucrose + 100  $\mu\text{M}$  acetosyringone (As) + 1.0 mg l<sup>-1</sup> ZET + 0.2 mg l<sup>-1</sup> IAA and co-cultivated in dark for two days in the culture room. Thereafter, The explants were incubated on SIM-1 medium consisting of MS salts + 2 % (w/v) sucrose + 500 mg l<sup>-1</sup> cefotaxime + 1.0 mg l<sup>-1</sup> ZET + 0.2 mg l<sup>-1</sup> IAA for five days and then transferred to SIM-1 medium containing 250 mg l<sup>-1</sup> cefotaxime + 50 mg l<sup>-1</sup> kanamycin or 20 mg l<sup>-1</sup> hygromycin or 10 mg l<sup>-1</sup> basta for 21–30 days. The independently regenerated shoots with first pair of vegetative leaves developed on first selection cycle of either kanamycin or hygromycin or basta were identified, excised, and were directly sub-cultured on the selection medium SIM-2 having same constituents as SIM-1 and incubated for 21–30 days. The independent shoots that regenerated after the second successive selection cycle were sub-cultured on SEM containing MS medium with B5 vitamins + 2 % (w/v) sucrose + 1.0

mg l<sup>-1</sup> GA<sub>3</sub> + 50 mg l<sup>-1</sup> kanamycin or 20 mg l<sup>-1</sup> hygromycin or 10 mg l<sup>-1</sup> basta for 10–14 days.

The same procedure was followed for leaf-discs using 2.5 mg l<sup>-1</sup> BA + 0.5 mg l<sup>-1</sup> IAA and 3 % (w/v) maltose (HiMedia Labs, Mumbai, India) as carbon source in SIM-1 and SIM-2 (Koul *et al.*, 2012; Koul *et al.*, 2014). The shoots recovered on SEM medium from either explant type, having 2–3 leaves were transferred to RIM containing half-strength MS medium with B<sub>5</sub> vitamins + 2 % (w/v) sucrose + 0.5 mg l<sup>-1</sup> indole-3-butyric acid (IBA) + 50 mg l<sup>-1</sup> kanamycin or 20 mg l<sup>-1</sup> hygromycin or 10 mg l<sup>-1</sup> basta for 15 days. The percentage transformation efficiency was determined as independent transgenic events received after the second selection divided by total number of explants multiplied by 100.

#### *Hardening and acclimatization of regenerated plantlets*

The rooted plantlets with two or three pairs of vegetative leaves were removed from the culture tubes, washed properly in running water to remove traces of agar and transferred into plastic pots containing sterilized soilrite (Keltech Energies Ltd. Bengaluru, India), irrigated with half-strength liquid MS medium devoid of sucrose and incubated under high humidity in a growth chamber (Convion Adaptis 1000 PG, Canada) for 14 days as described earlier (Koul, 2013). After 14 days of hardening, the potted plants were transferred to 12 inch deep earthen pots filled with soil:sand:farmyard manure in the ratio 3:1:1 and transferred to a glasshouse maintained at 24 ± 1 °C under natural light for flowering and seed setting.

#### *β-glucuronidase activity assays*

Histochemical and fluorometric assays for β-glucuronidase activity in explants of tomato was performed according to Jefferson *et al.*, (1987). Excised cotyledon and leaf explants of tomato were inoculated with *A. tumefaciens* strain LBA4404 harbouring p35SGUS-INT followed by transient fluorometric GUS assay after agro-inoculation. Fluorometric assay for transient GUS activity in

cotyledon and leaf explants was performed after 7 days of agro-inoculation, while stable expression was estimated in the leaves of mature transgenic plants using the specific substrate 4-methyl umbelliferyl glucuronide (MUG; Sigma, USA). The GUS activity was expressed as nmol MU/mg protein/min. The aliquots of plant extracts (100 μl) were used for quantitation of total soluble protein (TSP) according to procedure of Bradford (1976). The histochemical assay of β-glucuronidase (GUS) activity in different explants of T<sub>0</sub> and T<sub>1</sub> transformants was performed according to Jefferson *et al.*, (1987). The explants were examined under the stereomicroscope to score and document the GUS foci.

#### *Polymerase chain reaction (PCR) and DNA blot hybridization*

Genomic DNA was isolated from fresh leaves of T<sub>0</sub> transgenic and non-transgenic control tomato plants using Gen Elute plant genomic DNA miniprep kit according to manufacturer's instructions (Sigma, USA). PCR analysis of genomic DNA (100 ng) was achieved by amplification of 506 bp fragment of *uidA* gene in the Gene Amp<sup>®</sup> PCR system 9700 (PE Biosystems, USA) using the set of specific primers for 30 cycles and each cycle comprising of denaturation at 92 °C for 1.5 min, annealing at 58 °C for 1 min followed by extension at 68 °C for 3 min. The amplified products were electrophoresed on 1.5 % (w/v) agarose gel and visualized after staining in 0.5 mg l<sup>-1</sup> ethidium bromide solution on a UV transilluminator.

DNA blot hybridization was performed to confirm the stable integration of the transgene in the transgenic plants according to the procedure of Sambrook and Russell (2001). Approximately 10 μg of tomato genomic DNA from the transgenic and non-transgenic control plants was digested for 16–24 h with *EcoRI* (New England Biolabs, USA), that cuts the T-DNA of p35SGUS-INT vector at one site. The digested genomic DNA was resolved in 0.8 % (w/v) agarose gel and immobilized on BioBond Plus nylon membrane (Sigma, USA) by vacuum transfer (Vacuum blotter model 785, BIO-RAD, USA). The

blots were hybridized at 58 °C for 24 h with 2.02 kb *Bam*HI-*Sst*I fragment of *uidA* gene from p35SGUS-INT, and radiolabelled with  $\alpha^{32}$ PdCTP (BRIT, Mumbai India). Blots were washed under stringent conditions (high temperature and low salt concentration of buffer) and exposed to Fuji screen for 48 h followed by scanning and documentation on Typhoon Trio Plus phosphoimager (GE Healthcare Life Sciences AB, Sweden).

#### Reverse transcriptase PCR (RT-PCR) analysis

Total RNA was isolated from leaf and seed tissues using TRI reagent (Sigma, USA) and treated with RNase-free DNaseI (Sigma, USA). The cDNA first-strand was synthesized by enhanced Avian RT-PCR kit (Sigma, USA). Reverse transcription was performed at 50 °C for 10 min, initial denaturation at 95 °C for 5 min followed by 40 amplification cycles comprising of 10 s denaturation at 95 °C and combined annealing and extension for 30 s at 60 °C in 25  $\mu$ l reaction volume, according to manufacturer's instructions. PCR amplification of cDNA was performed using the specific primers of *nptII*, *uidA*, *cry1Ab*, *ai-PI*, *taf4b*, *nbri12* and *gfp-nbri12* genes. The set of primers for these genes and their product size are shown in Table 1.

#### Statistical analysis

Each experiment was performed with an average of 150 explants per treatment and repeated three times. Tissue culture data was subjected to analysis of variance by One-Way ANOVA to detect the significance of differences among treatment means using Duncan's Multiple Range Test at  $P < 0.05$ . T<sub>1</sub> seeds were germinated on MS basal + 50 mg l<sup>-1</sup> kanamycin or 20 mg l<sup>-1</sup> hygromycin or 10 mg l<sup>-1</sup> basta medium and subjected to a  $\chi^2$  fitness test for progeny segregation to compare the expected and observed values.

## Results and discussion

#### Optimization of tomato regeneration

We have optimized the procedure for *in vitro* regeneration using cotyledon and leaf explants complementary to efficient *Agrobacterium*-mediated

transformation for the development of transgenic plants in commercial cultivars of tomato.

#### Effect of explant type and hormone supplement

Selection of suitable explants for *Agrobacterium* infection is the first and crucial step to complement high-frequency of transformation. Explant age is a crucial parameter, as it profoundly influences the success of *in vitro* regeneration, development and compatibility for higher transformation efficiencies (Bhatia *et al.*, 2004). There are several reports of tomato transformation using cotyledons from 6–10 day old seedlings (Frary and Earle, 1996; Park *et al.*, 2003; Sun *et al.*, 2006; Kaur and Bansal, 2010; Rai *et al.*, 2012; Namitha and Negi, 2013) and vegetative leaves of 16–20 day old plantlets (Thi Van *et al.*, 2011; Khuong *et al.*, 2013; Koul *et al.*, 2014).

The leaf discs prepared from 16–18 days old leaves having a portion of midrib were incubated for 20 to 25 days on MS basal supplemented with 3% maltose and fortified with combination of different cytokinins 6-benzyladenine (BA), zeatin (ZET), kinetin (KIN), 2-isopentyladenine (2-iP) and thidiazuron (TDZ) with 0.5 mg l<sup>-1</sup> IAA. The leaf discs (2–5 mm) prepared from middle-distal portion of leaf and their abaxial orientation on agar-medium showed a maximum percent response of 75.60% with 4.30 shoots per responding explant. Whereas, the -discs prepared from proximal region of leaf showed 60.30% response with 3.26 shoots per responding explants, on media supplemented with 2.0 mg l<sup>-1</sup> BA + 0.5 mg l<sup>-1</sup> IAA (Fig 2A and supplementary table 1). In a similar experiment with 10-day-old cotyledon explants (8 mm in length), a maximum percent response of 75.93% with 3.26 shoots per responding explant was obtained on media supplemented with 1.0 mg l<sup>-1</sup> zeatin + 0.2 mg l<sup>-1</sup> IAA (Fig 2A and supplementary table 2).

The individual shoots developed on SIM medium were excised and sub-cultured on shoot elongation medium (SEM) consisting of MS medium fortified with different concentrations of GA<sub>3</sub> ranging from 0.5 to 3.0 mg l<sup>-1</sup> and 1.0 mg l<sup>-1</sup> GA<sub>3</sub> showed maximum

frequency of shoot elongation within 15 days of incubation and also decreased the emergence of rosette-like leafy shoots. The elongated shoots were healthy with 2 to 3 pair of vegetative leaf (Fig 2J and supplementary Fig. 1).

The response to RIM supplemented with different auxins (IAA, IBA and NAA) was also determined for induction of roots on regenerated shoots. RIM

supplemented with 0.5 mg l<sup>-1</sup> IBA was found optimal for 100% root induction with an average of 13.18 roots per responding shoot after 15 days of incubation (Fig 2K and supplementary table 3). The *in vitro* grown rooted plantlets were potted in soilrite mixture and initially acclimatized under high humidity, in growth chamber and thereafter, the plantlets were transferred to contained glasshouse for normal growth and development (Fig 2L, M).

**Table 1.** Profile of various genes used for the transformation of PED cultivar.

Plasmid vector	Target gene	Forward and reverse primers (5'-3')	Site in gene (bp)	Amplicon size (bp)	Transformation efficiency (%)	
					Cotyledon* (% ± SE)	Vegetative leaf* (% ± SE)
p35SGUS-INT	<i>npII</i> <sup>1</sup>	TATTCGGCTATGACTTGGGC GCGAACGCTATGCCTGATA	201-879	678	-	-
p35SGUS-INT	<i>uidA</i> <sup>2</sup>	TTTAACTATGCCGGGATCCATCGC CCAGTCGAGCATCTCTCAGCGTA	789-1295	506	18.80 ± 1.0	35.70 ± 1.2
pBIN200	<i>cry1Ab</i> <sup>3</sup>	TGGTACAACACTGGCTTGGGA ATGGGATTGGGTGATTGTA	634-1432	800	21.38 ± 1.34	34.74 ± 0.99
pPAK	$\alpha_1$ -PI <sup>4</sup>	GAAGATCCTCAAGGAGATGCTGC CTTCTGAGTAGGGTTAACACCTT	1-1182	1182	3.17 ± 0.63	25.84 ± 0.91
pBIN442	<i>taf4b</i> <sup>5</sup>	GCTCTAGAATGGATCTCTCCATTGTCAAGCTC CGAGCTCAACATCCGAGCAGATTCTATTGTATA	1-2163	2163	21.26 ± 0.79	29.59 ± 0.96
pBIN1612	<i>nbri12</i> <sup>6</sup>	CACCATGGCAGCATGGAGACCCC GGTGGTAGAGTGGAGAGAGAGG	1-568	568	18.38 ± 1.82	28.95 ± 1.68
pBIN1613	<i>gfp-nbri12</i> <sup>7</sup>	CACCATGGCAGCATGGAGACCCC GGTGGTAGAGTGGAGAGAGAGG	1-568	568	9.83 ± 1.04	21.83 ± 0.99

\*Values are the means (number of transformation experiments = 3). Minimum of 150 leaf disc explants were used for each transformation experiment ( $P < 0.0001$ ).

<sup>1</sup>GUS reporter gene from *Escherichia coli* strain K-12, encoding  $\beta$ -glucuronidase protein

<sup>2</sup>Coding region of neomycin phosphotransferase gene from *Escherichia coli* strain K-12

<sup>3</sup>Crystal protein toxin gene from *Bacillus thuringiensis* (Lab. construct)

<sup>4</sup>Human alpha-1-proteinase inhibitor gene (Jha *et al.*, 2012)

<sup>5</sup>TATA box-binding protein associated factor gene from *Arabidopsis thaliana* (Sawant *et al.*, 2010)

<sup>6</sup>Chitinolytic gene from a fern *Tectaria*, encodes a protein toxic to whitefly *Bemisia tabaci* (Singh *et al.*, 2011)

<sup>7</sup>Green fluorescent protein and chitinolytic gene fusion (Singh *et al.*, 2011).

#### Effect of carbon source

Different concentrations of sucrose and maltose were tested as carbon sources along with varying concentrations of cytokinins (BA and ZET) for direct regeneration from leaf discs. Incubation of leaf discs on SIM supplemented with 2.5 mg l<sup>-1</sup> BA + 0.5 mg l<sup>-1</sup> IAA and 3 % (w/v) maltose, showed a maximum of 96.2 % response with 5.8 mean numbers of elongated shoots per responding explant in PED variety of

tomato (Table 2). A similar test was performed to determine maximum regeneration from cotyledon explant on medium with 1.0 mg l<sup>-1</sup> ZET and IAA 0.2 mg l<sup>-1</sup>. It was determined that 2 % (w/v) sucrose showed a maximum of 75.93 % responding explants with 3.26 mean numbers of elongated shoots per responding explant (Table 2).

Sucrose (3 %) is the most commonly used and preferred carbon source in plant tissue culture

medium for wide range of crop plants including tomato (McCormick *et al.*, 1986; George, 1993; Bhatia *et al.*, 2004). However, use of decreased concentration of sucrose or its substitution with maltose, has shown improved frequency of direct organogenesis and recovery of elongated shoots in different cultivars of tomato, as reported earlier (El-

Bakry *et al.*, 2002; Steinitz *et al.*, 2006; Koul *et al.*, 2012). This may be due to rapid absorption and metabolism of maltose for induction and formation of well organized meristem in developing shoot buds and shoots, while suppressing the formation of disorganized shoot apical meristem (El-Bakry *et al.*, 2002; Bhatia *et al.*, 2004).

**Table 2.** Effect of carbon source and plant growth regulators combinations on shoot regeneration from cotyledon and vegetative leaf explants in PED cultivar.

Cotyledon explants				Vegetative leaf explants			
Carbon source (%)	PGR (mg l <sup>-1</sup> )	Responding explants (%)	Elongated shoots per responding explant	Carbon source (%)	PGR (mg l <sup>-1</sup> )	Responding explants (%)	Elongated shoots per responding explant
1 % Sucrose	1.0 ZET + 0.2 IAA	16.00 <sup>a</sup>	0.96 <sup>a</sup>	2 % Sucrose	1.0 BA + 0.5 IAA	55.26 <sup>a</sup>	2.70 <sup>ab</sup>
2 % Sucrose	1.0 ZET + 0.2 IAA	75.93 <sup>c</sup>	3.26 <sup>c</sup>	2 % Sucrose	2.5 BA + 0.5 IAA	57.00 <sup>a</sup>	2.93 <sup>b</sup>
3 % Sucrose	1.0 ZET + 0.2 IAA	55.73 <sup>b</sup>	1.66 <sup>ab</sup>	2 % Sucrose	1.0 ZET + 0.5 IAA	56.10 <sup>a</sup>	2.98 <sup>b</sup>
1 % Maltose	1.0 ZET + 0.2 IAA	12.66 <sup>a</sup>	0.66 <sup>a</sup>	2 % Sucrose	2.5 ZET + 0.5 IAA	49.26 <sup>a</sup>	1.95 <sup>ab</sup>
2 % Maltose	1.0 ZET + 0.2 IAA	56.80 <sup>b</sup>	1.36 <sup>a</sup>	3 % Maltose	1.0 BA + 0.5 IAA	83.73 <sup>b</sup>	1.57 <sup>a</sup>
3 % Maltose	1.0 ZET + 0.2 IAA	65.30 <sup>bc</sup>	2.55 <sup>bc</sup>	3 % Maltose	2.5 BA + 0.5 IAA	96.20 <sup>b</sup>	5.80 <sup>c</sup>
				3 % Sucrose	2.5 BA + 0.5 IAA	87.28 <sup>b</sup>	4.75 <sup>c</sup>
				3 % Maltose	1.0 ZET + 0.5 IAA	60.00 <sup>a</sup>	2.20 <sup>ab</sup>
				3 % Maltose	2.5 ZET + 0.5 IAA	92.30 <sup>b</sup>	2.70 <sup>ab</sup>

Duncan's Multiple Range Test (DMRT) was performed (\*\* $P < 0.05$ ), PGR Plant growth regulator.

#### Optimization of tomato transformation procedure

Several parameters that profoundly influence T-DNA transfer were optimized to improve *Agrobacterium*-mediated transformation and recovery of stable transgenic plants. Excised cotyledon and leaf explants

were inoculated with *A. tumefaciens* strain LBA4404 harbouring p35SGUS-INT followed by transient GUS assay after seven days, while stable expression was estimated in the mature transgenic plants.

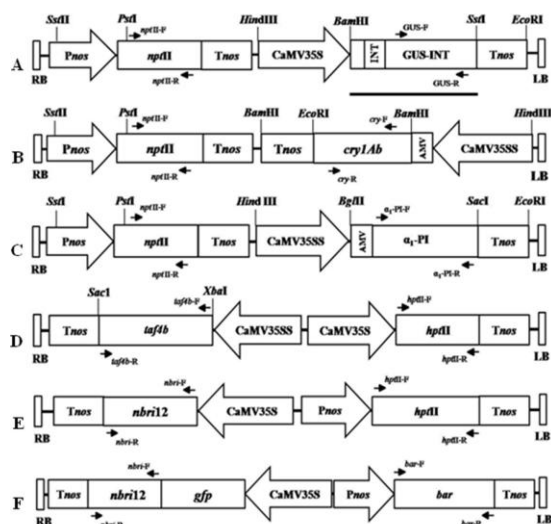
**Table 3.** Segregation analysis of T<sub>1</sub> transgenic tomato seeds developed with p35SGUS-INT vector.

T <sub>1</sub> transgenic plants	Response of seeds on kanamycin selection medium			$\chi^2$ value <sup>a</sup>
	Total	Kan <sup>r</sup>	Kan <sup>s</sup>	
GI 1a	68	50	18	0.08
GI 1b	22	18	4	0.00
GI 5a	28	23	5	0.76
GI 9f	57	42	15	0.05
GI 4c	64	47	17	0.08
GI 5e	36	29	7	0.58
GI 8f	76	64	12	3.44
GI 5d	21	14	7	0.78

<sup>a</sup>  $\chi^2_1 = 3.841$  at  $P \leq 0.05$

Kan<sup>r</sup> Kanamycin-resistant, Kan<sup>s</sup> Kanamycin-sensitive.

Results obtained with the optimization of different parameters like pre-incubation duration of explants, *Agrobacterium* cell density, co-cultivation period, vacuum treatment, supplementation of acetosyringone and sonication treatment are graphically summarized in Fig. 3. These results were the same in the both type of explants.



**Fig. 1.** T-DNA region of binary vectors used for tomato transformation. **(A)** p35SGUS-INT harbouring *uidA* gene driven by *CaMV35S* promoter with a 190 bp intron, **(B)** pBIN200 harbouring *cry1Ab* gene driven by the *CaMV35SS* promoter, **(C)** T-DNA region of pPAK harbouring modified  $\alpha_1$ -PI gene driven by *CaMV35SS* promoter, **(D)** T-DNA region of pBIN442 with 2,160 bp *taf4b* gene driven by *CaMV35SS* promoter, **(E)** T-DNA region of pBIN1612 with 568 bp *nbri12* gene driven by *CaMV35S* promoter, **(F)** T-DNA region of pBIN1613 with 1,351 bp *gfp-nbri12* gene driven by *CaMV35S* promoter. RB– right border, Pnos–nopaline synthase promoter, *CaMV35SS*– *CaMV35S* promoter with double enhancer, *nptII*–neomycin phosphotransferase gene,  $\alpha_1$ -PI– modified human  $\alpha_1$ -proteinase inhibitor gene, *taf4b*– TATA box-binding protein associated factor gene of the basal transcription initiation complex, *nbri12*– chitinolytic protein toxin gene, *hptII*–hygromycin phosphotransferase gene, *bar*– *bar* gene conferring resistance to basta (glufosinate), Tnos–nopaline synthase terminator, AMV– avian mosaic virus 5' UTR, LB– left border. Different PCR primers used in the present study are shown with arrows

indicating their binding positions. The bold line below GUS-INT gene shows *Bam*HI and *Sac*I fragment of *uidA* (GUS) used as radiolabelled probe.

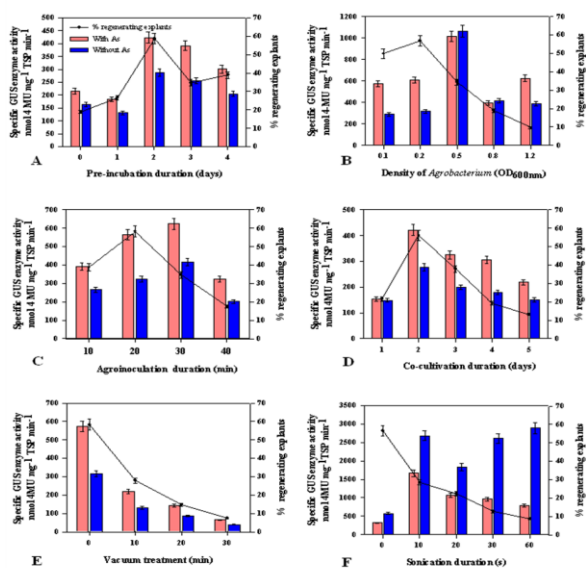
Transient GUS assay data presented in Fig 3A shows a positive co-relation of three day pre-incubation with maximum expression of GUS enzyme activity. Overnight grown *Agrobacterium* culture was resuspended in liquid co-cultivation medium (MS medium with pH 5.6), to different OD<sub>600</sub> ranging from 0.1–1.2 for co-cultivation with leaf and cotyledon explants. *Agrobacterium* cell density of  $2 \times 10^9$  cells ml<sup>-1</sup> at OD<sub>600</sub> 0.2–0.25 was found optimal for maximum response based on transient assay of GUS activity and subsequent development of shoot buds and number of shoots per responding explant (Fig 3B).



**Fig. 2.** Tomato regeneration and *Agrobacterium*-mediated transformation of cotyledon and leaf explants. **(A)** Excision of cotyledon (upper panel) and leaf (lower panel) for explant preparation. The black arrow indicates the position of the forceps and the dotted lines (yellow) indicate the trimming of the leaf margins with a sharp scalpel blade (step 1 and 3 for cotyledon; step 1, 2, 3 and 8 for leaf). The solid lines

(yellow) indicates the preparation of leaf discs prior to agroinoculation (step 2 for cotyledon; step 4, 5, 6 and 7 for leaf), **(B)** Regenerating leaf discs on SIM-1, **(C)** Shoot induction on SIM-2 medium, **(D-F)** Stages during selection and screening showing the non-transformed escapes, chimeric and regenerating transformed shoots, **(G, H)** Occurrence of leafy shoots, **(I)** Explant bearing true shoot (the dotted line indicates the location for excision of shoot for SEM), **(J)** Elongation of *in vitro* regenerated shoot, **(K)** Root induction, **(L)** Hardening of plantlets, **(M)** Fruit setting in glasshouse, **(N)** Single selection cycle resulting in chimeric GUS expression in tomato leaf, **(O)** Uniform GUS expression in tomato leaf, **(P)** in roots, **(Q)** in T.S. of fruit from stable transgenic plant using modified procedure involving double selection cycle.

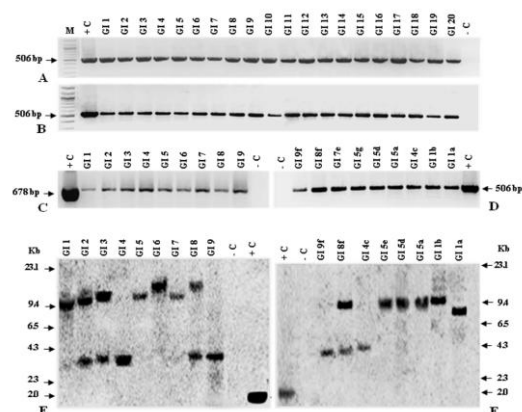
Period of agroinoculation was varied from 10–40 min in liquid medium and a 20 min time duration was found to be optimal (Fig 3C). Agroinoculated explants were co-cultivated for different durations (1–5 days) in dark on solid SIM medium containing 100  $\mu$ M acetosyringone (As). The percent response of regenerating explants was higher for both type of explants co-cultivated for two days and a longer duration adversely affected the number of shoot buds and shoots per responding explant (Fig 3D).



**Fig. 3.** Effect of various parameters on agroinoculation of leaf discs based on *in vitro* regeneration after transformation and transient GUS activity. **(A)** Duration of pre-incubation of explants

on acetosyringone, **(B)** OD<sub>600</sub> of *Agrobacterium* suspension, **(C)** Duration of agroinoculation, **(D)** Co-cultivation duration, **(E)** Vacuum treatment, **(F)** Sonication treatment during agroinoculation.

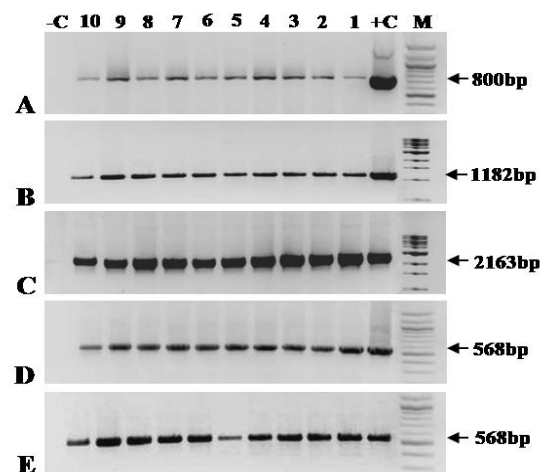
Vacuum treatment to the agro-treated explants was found to be inhibitory for T-DNA transfer and maximum GUS expression was recorded in the absence of vacuum treatment (Fig 3E). Sonication for 10 s in the absence of acetosyringone showed maximum GUS activity, while a longer duration of sonication in general had a detrimental effect on the regeneration response for both the explant type (Fig 3F). The supplementation of acetosyringone significantly increased the transient GUS expression and showed positive interaction with all the parameters, except vacuum and sonication treatment. Pre-incubation of tomato leaves or cotyledons on SIM for 2–3 days before co-cultivation with *Agrobacterium* seems to enhance the transformation process as shown earlier in different plant species (Sangwan *et al.*, 1992; Sunilkumar *et al.*, 1999). This may be attributed to initiation of active cell division, sufficient production of *vir*-inducing compounds during incubation that stimulates and facilitates *Agrobacterium* attachment onto the newly synthesized cell wall. The optimal temperature for agro-inoculation was found to be  $24 \pm 1$  °C, which is consistent with several earlier investigations (Jin *et al.*, 1993; Baron *et al.*, 2001). This optimal temperature allows maximum induction of *vir* genes and mobilization of T-DNA into plant cells (Gelvin, 2003; Tzfira and Citovsky, 2002, 2006).



**Fig. 4.** Molecular characterization of T<sub>0</sub> and respective T<sub>1</sub> progeny of transgenic tomato plants

transformed with vector p35SGUS-INT. **(A)** PCR analysis of T<sub>0</sub> transgenic plants with *uidA* gene-specific primers, lanes 3–22 of plant no. GI1–GI 20, **(B)** RT-PCR analysis of T<sub>0</sub> transgenic plants with *uidA* gene-specific primers, lanes 3–22 of plant no. GI1–GI20, lane +C – plasmid DNA of p35SGUS-INT showing an amplicon size of 506 bp as positive control and lane -C – cDNA amplification from untransformed tomato plant as negative control showing no amplicon, **(C)** RT-PCR analysis of T<sub>0</sub> transgenic plants with *nptII* gene-specific primers, lanes 2–10 of plant no. GI1–GI9, lane +C – plasmid DNA of p35SGUS-INT showing an amplicon size of 678 bp as positive control and lane -C – cDNA amplification from untransformed tomato plant as negative control showing no amplicon **(E, F)** DNA blot hybridization of T<sub>0</sub> (lane 1–9) and T<sub>1</sub> transgenic plants (lane 1–9) probed with 2.02 kb *Bam*HI/*Sst*I *uidA* gene fragment isolated from p35SGUS-INT. Lane -C – genomic DNA from untransformed tomato plant and lane +C – 2.02 kb *Bam*HI/*Sst*I *uidA* gene fragment, **(D)** RT-PCR analysis of T<sub>1</sub> progeny with *uidA* gene-specific primers, lane +C – plasmid DNA of p35SGUS-INT binary vector showing an amplicon size of 506 bp and lane -C – cDNA amplification from untransformed tomato plant.

*Agrobacterium* infection leads to an early induction of defense genes in the host cell whereas, in later stages of infection, expression of other genes important for cell division are required for the successful transformation (Tzfira and Citovsky, 2002; Veena *et al.*, 2003; Wroblewski *et al.*, 2005). Supplementation of 100 µM acetosyringone during co-cultivation for two days with *Agrobacterium* cell density between  $2 \times 10^9$  cells ml<sup>-1</sup>, seems to offer optimum conditions for maximum transfection of excised somatic cells in both explant type. Whereas, co-cultivation of the explants with high density of *Agrobacterium* for longer duration, proved to be detrimental for survival of explants owing to the high multiplicity of infection and virulence response of bacterial cells, particularly to young dividing cells and tissues (Sangwan *et al.*, 1992; Gelvin, 2003).



**Fig. 5.** RT-PCR analysis of T<sub>0</sub> plants transformed with plasmid vectors **(A)** pBIN200 (*cry1Ab*), **(B)** pPAK ( $\alpha$ -1-PI), **(C)** pBIN442 (*taf4b*), **(D)** pBIN1612 (*nbri12*), **(E)** pBIN1613 (*gfp-nbri12*), lane M – 1Kb ladder in panel **(B, C)** and 100 bp ladder in panel **A, D, E**, lane +C – amplification of plasmid DNA of binary vector in panel **(A)** pBIN200, **(B)** pPAK, **(C)** pBIN442, **(D)** pBIN1612, **(E)** pBIN1613 respectively using gene specific primers and lane -C sample of cDNA from untransformed control plant used as negative control showing no amplicon.

The optimized conditions of *Agrobacterium* co-cultivation and *in vitro* selection of transformants in six common Indian cultivars of tomato were in the order PED > Gaurav > Pusa Ruby > S-22 > Pusa120 > Pusa hybrid 1 respectively (Supplementary table 4). Among these cultivars, PED reflected the maximum transformation efficiency of 35.70 % and was employed further to raise transgenics with six different genes of diverse traits, in order to study the efficacy of transformation procedure and performance of the optimized parameters (Supplementary table 4).

The concentration of different selection agents were also optimized with leaf-discs as shown in supplementary Fig. 2. *In vitro* culture of agroinoculated discs on threshold concentration of kanamycin (50 mg l<sup>-1</sup>) or hygromycin (20 mg l<sup>-1</sup>) or basta (10 mg l<sup>-1</sup>) have shown the development of three types of shoots; 1) partially bleached with pale green leaves as non-transformants, 2) showing partial

or sectorial bleaching possibly chimeric and 3) green, non-bleached shoots with healthy green leaves as putative transformants (Fig 2D–F). The green leaves of the third type of shoots were excised into small pieces and sub-cultured further on SIM selection medium for the generation of non-chimeric and true transgenic plants.

#### *Regeneration of T<sub>0</sub> tomato transformants*

The healthy green shoot primordia with first pair of green leaves developed on first selection medium SIM–1 after 21–30 days of incubation were cut again into leaf discs and sub-cultured directly onto SIM–2 supplemented with 2.5 mg l<sup>-1</sup> BA, 0.5 mg l<sup>-1</sup> IAA and 50 mg l<sup>-1</sup> kanamycin or 20 mg l<sup>-1</sup> hygromycin or 10 mg l<sup>-1</sup> basta (Fig 2B). Sub-culturing of these leaf discs in shoot induction medium (SIM) showed significant improvement in shoot induction and decreased the emergence of rosette-like leafy shoots and untransformed escapes (Fig 2C, I and supplementary Fig. 3). The rosette-like leafy shoot structures do not develop into normal plantlets and remain stunted (Fig 2G, H). The resultant shoots regenerated after the second selection cycle were thereafter transferred to SEM supplemented with 1.0 mg l<sup>-1</sup> GA<sub>3</sub> and suitable selection agents in MS basal medium. The individual elongated shoots were sub-cultured on RIM supplemented with selection agents that resulted into development of high-frequency rooted transgenic plantlets (90 to 100 %) in different cultivars (Fig 2K). The *in vitro* grown rooted plantlets after the initial acclimatization in the culture room or growth chamber under high humidity were transferred to glasshouse condition and showed normal flowering and fruit development (Fig 2M).

Prolonged culture of tomato explants on high BA-supplemented media has reflected the development of abnormalities including formation of undifferentiated structures owing to suppression of apical dominance, disruption of cytokinin polarity and differential patterns of gene expression during the development of shoot apical meristems (Fleming *et al.*, 1993; Vernoux *et al.*, 2000). In our study a combination of BA or zeatin with low concentration of IAA promoted

efficient shoot proliferation on maltose-fortified medium than on sucrose, as carbon source. This may be attributed to formation of proper cytokinin gradient and efficient utilization of BA or zeatin by the actively dividing cells on maltose sugar for development of adventitious shoot buds as demonstrated earlier (George, 1993; Bhatia *et al.*, 2004).

#### *Effect of hormones in SEM and RIM*

Sub-culturing of tomato shoot primordia on GA<sub>3</sub> supplemented medium has accelerated shoot elongation and resulted in a higher frequency of well developed shoots compared to the results with prolonged sub-culturing on low BA (Kaur and Bansal, 2010). Cytokinins like BA, promote shoot proliferation and prolonged incubation may cause shoots to develop with more than one apical meristem. GA<sub>3</sub> helps to preserve the integrity of apical buds and eliminates the formation of abnormal multi-apexed shoots which are routinely induced by combination of BA and IAA in several members of Solanaceae (George, 1993; Vernoux *et al.*, 2000). We have achieved maximum rooting with a low concentration of IBA and reduced concentration of sucrose in the medium as reported earlier (Frery and Earle, 1996; Kaur and Bansal, 2010; Rai *et al.*, 2012). The optimized parameters of *in vitro* regeneration showed compatible response with explants co-cultivated with *Agrobacterium* for shoot initiation, elongation and rooting. A schematic representation of the optimized time frame for tomato transformation and development of transgenic plants is shown in supplementary Fig. 4.

#### *β-glucuronidase activity assay*

The T<sub>0</sub> putative transformants developed with p35SGUS-INT vector revealed differential levels of β-glucuronidase activity confirmed by fluorometric assay ranging from 15.13 to 1568.17 nmol MU mg<sup>-1</sup> TSP min<sup>-1</sup>. The GUS expression values of nine T<sub>0</sub> transgenic plants are shown in supplementary Fig. 5. Quantitative GUS assay was performed at different developmental stages from flowering, fruit and seed setting stages in three T<sub>0</sub> transgenic plants (GI 1, GI 5

and GI 9) having single copy insertion of *uidA* gene. The analysis of data showed an increasing trend in expression level of GUS activity up to flowering stage followed by a slight decrease during fruit and seed setting stage as shown in supplementary Fig. 6.

Quantitative assay of GUS activity in eight independent T<sub>1</sub> transgenic plants, with single copy insertion of *uidA* transgene as evident from DNA blot hybridization (Fig 5D) consistently showed higher level of GUS expression (>175 nmol MU mg<sup>-1</sup> TSP min<sup>-1</sup>) whereas one T<sub>1</sub> plant (GI 8f) with two copies of the GUS gene, showed six fold lower level of GUS expression (data not shown). Histochemical assay for GUS expression in T<sub>1</sub> transgenic tomato plants showed uniformly dark blue colour indicating constitutive GUS expression in leaves, roots and fruit compared to chimeric GUS expression in leaves developed with single step selection procedure (Fig 2N–Q).

#### *Molecular characterization of T<sub>0</sub> transformants*

A total of 20 T<sub>0</sub> transgenic tomato plants transformed with p35SGUS-INT were considered for molecular analysis. PCR results of these transformants showed amplification of an anticipated 506 bp amplicon for *uidA* gene and were similar to the positive control (Fig 4A). RT-PCR analyses of these twenty transformants for *uidA* gene and nine selected transformants for *nptII* gene showed amplification of anticipated 506 bp and 678 bp amplicons of *uidA* and *nptII* gene transcripts, respectively (Fig 4B, C).

DNA blot hybridization of the nine T<sub>0</sub> transgenic plants with *uidA* gene probe revealed mostly single and few double copy integration of the transgene. DNA blot of eight randomly selected T<sub>1</sub> progenies of five T<sub>0</sub> plants, GI1, GI4, GI5, GI8 and GI9, showed strong hybridization signals with *uidA* gene probe. Transgenic plant number GIa, GI1b, GI5a, GI5d, GI5e, GI4c and GI9f showed single copy integration while plant GI8f showed the integration of two copies of the transgene (Fig 4E, F). RT-PCR analysis of these T<sub>1</sub> plants showed amplification of expected 506 bp

amplicon of *uidA* gene suggesting stable inheritance of the transgene (Fig 4D).

The inheritance pattern of *uidA* gene in T<sub>1</sub> progeny of primary transgenic plants was analyzed by germinating the transgenic tomato seeds on kanamycin-supplemented medium (50 mg l<sup>-1</sup>). Antibiotic screening of T<sub>1</sub> seeds followed by PCR analysis revealed segregation according to Mendelian ratio 3:1 (resistant:susceptible,  $p \leq 0.05$ ,  $\chi^2 = 3.841$ ) for kanamycin tolerance (Table 3). Single insertion of foreign gene in T<sub>0</sub> plants often leads to the expected 3:1 segregation ratio in selfed population. Except under rare circumstances, the segregation will deviate from the one-locus Mendelian segregation ratio. This may be attributed to segregation of two very close copies of transgenes at a single locus in T<sub>0</sub> plant. Our results for segregation analysis of T<sub>1</sub> progenies advocate stable integration, inheritance and high-level expression of *uidA* gene as the single-locus-insertion for dominant trait in the T<sub>0</sub> transgenic plants.

The T<sub>0</sub> transgenic plants developed with other plant vectors were also subjected to RT-PCR analyses and results of 10 randomly selected T<sub>0</sub> plants developed with pBIN200 (*Bt-cry1Ab*), pPAK ( $\alpha$ -PI), pBIN442 (*taf4b*), pBIN1612 (*nbri12*) and pBIN1613 (*gfp-nbri12*) showed anticipated 800 bp, 1182 bp, 2,163 bp, 568 bp and 568 bp amplicons for corresponding genes respectively (Fig 5A–E). The transcriptional stability of different transgenes verified the effectiveness of our transformation procedure.

#### *A comparative analysis of tomato transformation procedures*

McCormick *et al.*, (1986) had reported moderate transformation frequency in tomato using leaf disc explants and the application of a feeder layer for regeneration of transformants. Later, Frary and Earle (1996) reported tomato transformation using cotyledon explants exhibiting a transformation efficiency of 10.6 % for a commercial cultivar MoneyMaker. The same group reported the use of half-strength MS and 1 % sucrose as carbon source for

*in vitro* growth of seedlings from cotyledons and has also preferred the use of feeder layer in pre-incubation media. In our modified procedure, a maximum transformation efficiency of 21.38 % was observed with cotyledon explants and an enhanced transformation efficiency of 35.70 % with leaf explants using PED tomato cultivar. We have used a simple combination of MS salts and maltose in initial stage of development without the use of feeder layer for high recovery of transgenics. They have used 2.0 mg l<sup>-1</sup> zeatin as the only hormone for regeneration while a combination of 1.0 mg l<sup>-1</sup> zeatin and 0.2 mg l<sup>-1</sup> IAA was found most suitable for cotyledon explants in our investigation (Table 2). A different combination (0.5 mg l<sup>-1</sup> zeatin and 0.5 mg l<sup>-1</sup> IAA) with transformation efficiency of 37 % has been also been reported earlier in tomato for *uidA* gene using cotyledon explants (Kaur and Bansal, 2010). A combination of 8.9 μM 6-benzyladenine, 9.3 μM kinetin and 0.4 mg l<sup>-1</sup> thiamine and a very low concentration of kanamycin for the initial ten days on selection medium with cotyledon explants showing transformation efficiency of 44.3 % has been reported recently (Rai *et al.*, 2012). According to a recent report, 2.0 mg l<sup>-1</sup> BA and 0.1 mg l<sup>-1</sup> IAA was found optimal for shoot induction in hypocotyl and cotyledon explants (Namitha and Negi, 2013). Few recent reports have also shown maximum numbers of shoots on combination of 2.0 mg l<sup>-1</sup> BA + 1.0 mg l<sup>-1</sup> IBA (Janani *et al.*, 2013) and 1.0 mg l<sup>-1</sup> Zeatin-trans + 0.1 mg l<sup>-1</sup> IAA (Khuong *et al.*, 2013), using the vegetative leaf explants. It is interesting that none of these reports provide rigorous information regarding raising non-chimeric, true transgenic tomato plants. However, in the present study, the application of a second selection regime to the newly emerged leaf apart from routine sub-culturing on selection media with a combination of 2.5 mg l<sup>-1</sup> BA and 0.5 mg l<sup>-1</sup> IAA has resulted in higher yield of non-chimeric true transgenics possibly due to elimination of untransformed cells at a very early stage of regeneration.

To conclude, we have optimized a simple and improved tomato regeneration procedure that is

reproducible, genotype-independent and produces non-chimeric transformed shoots and has been extensively used for development of transgenic tomato plants. This optimized procedure for genetic transformation may be used for the introgression of different genes for improving the horticultural and agronomic traits in other commercial tomato cultivars.

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#### References

- Baron C, Domke N, Beinhofer M, Hapfelmeier S.** 2001. Elevated temperature differentially affects virulence, *vir* B protein accumulation and T-pilus formation in different *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains. *Journal of Bacteriology* **183**, 6852–686. <http://dx.doi.org/10.1128/JB.183.23.6852-6861.2001>
- Bergounoux V.** 2014. The history of tomato: From domestication to biopharming. *Biotechnology Advances* **32**, 170–189. <http://dx.doi.org/10.1016/j.biotechadv.2013.11.003>
- Bhatia P, Ashwath N, Senaratna T, Midmore D.** 2004. Tissue culture studies of tomato (*Lycopersicon esculentum*). *Plant Cell, Tissue and Organ Culture* **78**, 1–21. <http://dx.doi.org/10.1023/B:TICU.0000020430.08558.6e>
- Birch RG.** 1997. Plant transformation: Problems and strategies for practical applications. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 297–326. <http://dx.doi.org/10.1146/annurev.arplant.48.1.297>

- Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- Duclercq J, Sangwan-Norreel B, Catterou M, Sangwan RS.** 2011. De novo shoot organogenesis: from art to science. *Trends in Plant Science* **16**, 597–606.  
<http://dx.doi.org/10.1016/j.tplants.2011.08.004>
- EL-Bakry AA.** 2002. Effect of genotype, growth regulators, carbon source and pH on shoot induction and plant regeneration in tomato. *In Vitro Cellular & Developmental Biology - Plant* **38**, 501–507.  
<http://dx.doi.org/10.1079/IVP2002338>
- Famiani F, Ferradini N, Staffolani P, Standardi A.** 1994. Effect of leaf excision time and age, BA concentration and dark treatments on *in vitro* shoot regeneration of M26 apple rootstock. *Journal of Horticultural Science* **69**, 679–685.
- FAO Statistical Database.** 2012. FAOSTAT Agriculture data,  
<http://faostat.fao.org>.
- Fleming AJ, Mandel T, Roth I, Kuhlemeier C.** 1993. The pattern of gene expression in the tomato shoot apical meristem. *Plant Cell* **5**, 297–309.  
<http://dx.doi.org/10.1105/tpc.5.3.297>
- Frary A, Earle ED.** 1996. An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. *Plant Cell Reports* **16**, 235–240.  
<http://dx.doi.org/10.1007/BF01890875>
- Gao N, Shen W, Cao Y, Su Y, Shi W.** 2009. Influence of bacterial density during pre-culture on *Agrobacterium*-mediated transformation of tomato. *Plant Cell, Tissue and Organ Culture* **98**, 321–330.  
<http://dx.doi.org/10.1007/s11240-009-9566-2>
- Gelvin SB.** 2003. *Agrobacterium*-mediated plant transformation: the biology behind the “Gene Jockeying” tool. *Microbiology and Molecular Biology Reviews* **67**, 16–37.  
<http://dx.doi.org/10.1128/MMBR.67.1.16-37.2003>
- George EF.** 1993. Plant propagation by tissue culture, Vol. I, 2nd ed. The Technology, Exegetics Ltd, Edington Wilts.
- James C.** 2010. Global status of commercial biotech/GM crops: ISAAA Brief 42, Ithaca, NY, ISAAA.
- Janani C, Girija S, Ranjitha Kumar BD.** 2013. *In vitro* culture and *Agrobacterium* mediated transformation in high altitude tomato (*Lycopersicon esculentum* Mill.) cultivar Shalimar. *International Journal of Pharmacy Teaching & Practices* **4(1)**, 483–488.
- Jefferson RA, Kavanagh TA, Bevan MW.** 1987. GUS fusions: beta glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**, 3901–3907.
- Jha S, Agarwal S, Sanyal I, Jain GK, Amla DV.** 2012. Differential subcellular targeting of recombinant human  $\alpha_1$ -proteinase inhibitor influences yield, biological activity and in planta stability of the protein in transgenic tomato plants. *Plant Science* **196**, 53–66.  
<http://dx.doi.org/10.1016/j.plantsci.2012.07.004>
- Jin S, Roitsch T, Ankenbauer RG, Gordon MP, Nester EW.** 1993. The regulatory Vir A protein of *Agrobacterium tumefaciens* does not function at elevated temperature. *Journal of Bacteriology* **175**, 6830–6835.  
<http://jb.asm.org/content/175/21/683>
- Kaur P, Bansal KC.** 2010. Efficient production of transgenic tomatoes via *Agrobacterium*-mediated transformation. *Biologia Plantarum* **54**, 344–348.  
<http://dx.doi.org/10.1007/s10535-010-0060-9>
- Khuong TTH, Cre´te´ P, Robaglia C, Caffarri S.** 2013. Optimization of tomato Micro-tom regeneration and selection of glufosinate/Basta and dependency of gene silencing on transgene copy number. *Plant Cell Reports* **32**, 1441–1454.  
<http://dx.doi.org/10.1007/s00299-013-1456-8>
- Koul B, Srivastava S, Sanyal I, Tripathi BN, Sharma V, Amla DV.** 2014. Transgenic tomato line expressing modified *Bacillus thuringiensis cry1Ab* gene showing complete resistance to two lepidopteran pests. *SpringerPlus* **3**, 84–96.  
<http://dx.doi.org/10.1186/2193-1801-3-84>
- Koul B, Yadav R, Sanyal I, Sawant S, Sharma V, Amla DV.** 2012. *Cis*-acting motifs in artificially synthesized expression cassette leads to enhanced transgene expression in tomato (*Solanum lycopersicum* L.). *Plant Physiol Biochem* **61**, 131–141.  
<http://dx.doi.org/10.1016/j.plaphy.2012.09.014>
- Koul B.** 2013. Expression of insecticidal toxin coded by modified full-length and truncated Bt-*cry 1Ac* genes in transgenic tomato for assessment of their

stability and efficacy against target insects. PhD thesis, Banasthali Vidyapith, Rajasthan, India, p. 60–65.

**Li C-W, Lee S-H, Chan M-T.** 2013. Utilization of the plant methionine sulfoxide reductase B genes as selectable markers in Arabidopsis and tomato transformation. *Plant Cell, Tissue and Organ Culture* **113**, 555–563.

<http://dx.doi.org/10.1007/s11240-013-0296-0>

**Liénard D, Sourrouille C, Gomord V, Faye L.** 2007. Pharming and transgenic plants. *Biotechnology Annual Review* **13**, 115–147.

[http://dx.doi.org/10.1016/S1387-2656\(07\)13006-4](http://dx.doi.org/10.1016/S1387-2656(07)13006-4)

**Mathews H, Dewey V, Wagoner W, Bestwick RK.** 1998. Molecular and cellular evidence of chimaeric tissues in primary transgenics and elimination of chimaerism through improved selection protocols. *Transgenic Research* **7**, 123–129.

<http://dx.doi.org/10.1023/A:1008872425917>

**McCormick S, Jeanne N, Joyce F, Arlene B, Robert H, Robert F.** 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. *Plant Cell Reports* **5**, 81–84.

<http://dx.doi.org/10.1007/BF00269239>

**Murashige T, Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.

<http://dx.doi.org/10.1111/j.13993054.1962.tb08052.x>

**Namitha KK, Negi PS.** 2013. Morphogenetic potential of tomato (*Lycopersicon esculentum*) cv. 'Arka Ahuti' to plant growth regulators. *Notulae Scientia Biologicae* **5(2)**, 220–225.

**Park JR, Mc Farlane I, Phipps RH, Ceddia G.** 2011. The role of transgenic crops in sustainable development. *Plant Biotechnology Journal* **9**, 2–21.

<http://dx.doi.org/10.1111/j.1467-7652.2010.00565.x>

**Park SH, Morris JL, Park JE, Hirschi KD, Smith RH.** 2003. Efficient and genotype independent *Agrobacterium*-mediated tomato transformation. *Journal of Plant Physiology* **160**, 1253–1257.

<http://dx.doi.org/10.1078/0176-1617-01103>

**Rai GK, Rai NP, Kumar S, Yadav A, Rathaur S, Singh M.** 2012. Effects of explant age, germination medium, pre-culture parameters, inoculation medium, pH, washing medium and selection regime

on *Agrobacterium*-mediated transformation of tomato. *In Vitro Cell Developmental Biology-Plant* **48**, 565–578.

<http://dx.doi.org/10.1007/s11627-012-9442-3>

**Sambrook J, Russell DW.** 2001. Molecular cloning, A laboratory manual, Vol. 1–3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

**Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B.** 1992. Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. *Planta* **188**, 439–456.

<http://dx.doi.org/10.1007/BF00192812>

**Sato S.** 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature Letters* **485**, 635–641.

<http://dx.doi.org/10.1038/nature11119>

**Sawant SV, Srivastava R, Srivastava M, Singh PK, Srivastava S, Verma PC, Koul B, Shukla AK, Nautiyal CS, Amla DV.** 2010. A method for developing pathogen tolerant transgenic plants. Indian Patent No. 3177/DEL/2010.

**Singh PK, Upadhyay SK, Krishnappa C, Sharad S, Singh R, Rai P, Singh H, Mishra M, Singh AP, Verma PC, Pillai NK, Tuli R.** 2011. A process preparation of a novel insecticidal chitinase toxic against whiteflies, its encoding nucleotide and application thereof. Indian Patent No. 3851/DEL/2011.

**Steinitz B, Amitay A, Gaba V, Tabib Y, Keller M, Levin I.** 2006. A simple plant regeneration-ability assay in a range of *Lycopersicon* species. *Plant Cell, Tissue and Organ Culture* **84**, 269–278.

<http://dx.doi.org/10.1007/s11240-005-9032-8>

**Sun HJ, Uchii S, Watanabe S, Ezura H.** 2006. A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiology* **47**, 426–431.

<http://dx.doi.org/10.1093/pcp/pci251>

**Sunilkumar G, Vijayachandra K, Veluthambi K.** 1999. Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. *Plant Science* **141**, 51–58.

[http://dx.doi.org/10.1016/S0168-9452\(98\)00228-3](http://dx.doi.org/10.1016/S0168-9452(98)00228-3)

**Thi Van D, Ferro N, Jacobson H-J.** 2011. Development of a simple and effective protocol for

*Agrobacterium tumefaciens* mediated leaf disc transformation of commercial tomato cultivars. *GM Crops* **1**, 315–321.

<http://dx.doi.org/10.4161/gmcr.1.5.14703>

**Tzfira T, Citovsky V.** 2000. From host recognition to T-DNA integration: the function of bacterial and plant genes in the *Agrobacterium*-plant cell interaction. *Molecular Plant Pathology* **1**, 201–212.

<http://dx.doi.org/10.1046/j.13643703.2000.00026.x>

**Tzfira T, Citovsky V.** 2002. Partners in infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends in Cell Biology* **12**, 121–129.

[http://dx.doi.org/10.1016/S0962-8924\(01\)02229-2](http://dx.doi.org/10.1016/S0962-8924(01)02229-2)

**Tzfira T, Citovsky V.** 2006. *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology* **17**, 147–154.

[http://dx.doi.org/10.1016/S0962-8924\(01\)02229-2](http://dx.doi.org/10.1016/S0962-8924(01)02229-2)

**Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M.** 1990. Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Molecular and General Genetics* **220**, 245–250.

<http://dx.doi.org/10.1007/BF00260489>

**Veena Jiang H, Doerge RW, Gelvin SB.** 2003. Transfer of T-DNA and vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. *Plant Journal* **35**, 219–236.

<http://dx.doi.org/10.1046/j.1365-313X.2003.01796.x>

**Velcheva M, Faltin Z, Flaishman M, Eshdat Y, Perl A.** 2005. A liquid culture system for *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* L. Mill.). *Plant Science* **168**, 121–130.

<http://dx.doi.org/10.1016/j.plantsci.2004.07.037>

**Vernoux T, Kronenberger J, Grandjean O, Laufs P, Traas J.** 2000. PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem. *Development* **127**, 5157–5165.

**Wroblewski T, Tomczak A, Micheltore R.** 2005. Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato

and *Arabidopsis*. *Plant Biotechnology Journal* **3**, 259–273.

<http://dx.doi.org/10.1111/j.1467-7652.2005.00123.x>

**Zupan J, Muth TR, Draper O, Zambryski P.** 2000. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant Journal* **23**(1), 11–28.

<http://dx.doi.org/10.1046/j.1365-313X.2000.00808.x>

## Abbreviations

As	Acetosyringone
BA	6-Benzyladenine
<i>bar</i>	Basta-Glufosinate
<i>CaMV35S</i>	Cauliflower mosaic virus 35S double enhancer promoter
GA <sub>3</sub>	Gibberellic acid
<i>hpt</i>	Hygromycin phosphotransferase
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	α-Naphthalene acetic acid
<i>nos</i>	Nopaline synthase
<i>nptII</i>	Neomycin phosphotransferase
SIM	Shoot induction medium
SEM	Shoot elongation medium
RIM	Root induction medium
<i>uidA</i>	β-Glucuronidase

**Supplementary Table 1.** Effect of PGR's on leaf explants for shoot regeneration in tomato (*Solanum lycopersicum* L. var. PED).

PGR concentration (mg l <sup>-1</sup> )	% resp. explants	Elongated shoots per resp. explant	Leafy shoots per resp. explant	% resp. explants	Elongated shoots per resp. explant	Leafy shoots per resp. explant
BA 1.0	44.64 <sup>defg</sup>	2.47 <sup>bcd</sup>	3.65 <sup>cde</sup>	64.00 <sup>de</sup>	0.0 <sup>a</sup>	2.54 <sup>bcd</sup>
BA 2.0	60.30 <sup>g</sup>	3.26 <sup>d</sup>	1.90 <sup>abc</sup>	75.60 <sup>e</sup>	4.30 <sup>c</sup>	1.34 <sup>ab</sup>
BA 3.0	51.93 <sup>efg</sup>	1.00 <sup>ab</sup>	3.47 <sup>bcd</sup>	45.00 <sup>bcd</sup>	2.23 <sup>bc</sup>	3.20 <sup>bcd</sup>
ZET 1.0	40.08 <sup>cdefg</sup>	2.9 <sup>cd</sup>	2.23 <sup>bcd</sup>	49.00 <sup>bcd</sup>	2.03 <sup>bc</sup>	2.59 <sup>bcd</sup>
ZET 2.0	10.00 <sup>a</sup>	1.46 <sup>abc</sup>	0.00 <sup>a</sup>	22.50 <sup>ab</sup>	1.00 <sup>ab</sup>	0.00 <sup>a</sup>
ZET 3.0	10.00 <sup>a</sup>	1.00 <sup>ab</sup>	3.00 <sup>bcd</sup>	10.00 <sup>a</sup>	1.00 <sup>ab</sup>	3.00 <sup>bcd</sup>
KIN 2.0	20.00 <sup>abc</sup>	1.00 <sup>ab</sup>	2.36 <sup>bcd</sup>	27.43 <sup>abc</sup>	1.23 <sup>ab</sup>	2.93 <sup>bcd</sup>
KIN 5.0	50.73 <sup>efg</sup>	1.06 <sup>ab</sup>	2.46 <sup>bcd</sup>	50.66 <sup>cde</sup>	1.06 <sup>ab</sup>	2.46 <sup>bc</sup>
KIN 10.0	35.13 <sup>bcd</sup>	0.9 <sup>ab</sup>	2.43 <sup>bcd</sup>	40.76 <sup>bcd</sup>	1.16 <sup>ab</sup>	4.56 <sup>d</sup>
2-iP 2.0	30.70 <sup>abcde</sup>	0.00 <sup>a</sup>	1.80 <sup>abc</sup>	53.96 <sup>cde</sup>	1.16 <sup>ab</sup>	1.93 <sup>abc</sup>
2-iP 5.0	61.36 <sup>g</sup>	1.13 <sup>ab</sup>	2.60 <sup>bcd</sup>	51.80 <sup>cde</sup>	1.33 <sup>ab</sup>	3.66 <sup>cd</sup>
2-iP 10.0	14.93 <sup>ab</sup>	1.30 <sup>abc</sup>	1.23 <sup>ab</sup>	33.76 <sup>abc</sup>	1.43 <sup>ab</sup>	1.53 <sup>ab</sup>
TDZ 0.2	17.10 <sup>abc</sup>	1.30 <sup>abc</sup>	4.46 <sup>de</sup>	30.86 <sup>abc</sup>	1.53 <sup>ab</sup>	2.43 <sup>bc</sup>
TDZ 0.5	58.76 <sup>g</sup>	1.70 <sup>abcd</sup>	2.76 <sup>bcd</sup>	34.40 <sup>abc</sup>	1.46 <sup>ab</sup>	2.53 <sup>bcd</sup>
TDZ 1.0	24.76 <sup>abcd</sup>	0.00 <sup>a</sup>	5.56 <sup>e</sup>	61.53 <sup>de</sup>	1.03 <sup>ab</sup>	3.33 <sup>bcd</sup>

PGR Plant growth regulator, *resp.* Responding

BA 6-Benzyladenine, ZET Zeatin, KIN Kinetin, 2-iP 2-Isopentyladenine, TDZ Thidiazuron.

**Supplementary Table 2.** Effect of PGR's on cotyledon explants for shoot regeneration in tomato (*Solanum lycopersicum* L. var. PED).

PGR concentration (mg/l)	% Responding explants	Mean number of shoots per responding explant	
		Elongated shoots	Leafy shoots
		BA 1.0	42.33 <sup>bc</sup>
BA 2.0	67.28 <sup>d</sup>	2.80 <sup>d</sup>	1.20 <sup>a</sup>
BA 3.0	66.63 <sup>d</sup>	2.20 <sup>cd</sup>	5.34 <sup>fh</sup>
ZET 1.0	75.93 <sup>e</sup>	3.26 <sup>de</sup>	2.78 <sup>bcd</sup>
ZET 2.0	74.47 <sup>de</sup>	2.26 <sup>cd</sup>	3.73 <sup>de</sup>
ZET 3.0	68.33 <sup>d</sup>	1.18 <sup>b</sup>	5.47 <sup>gh</sup>
KIN 2.0	20.00 <sup>a</sup>	1.20 <sup>b</sup>	2.4 <sup>bc</sup>
KIN 5.0	37.49 <sup>bc</sup>	1.06 <sup>ab</sup>	4.93 <sup>efgh</sup>
KIN 10.0	26.5 <sup>b</sup>	0.00 <sup>a</sup>	7.4 <sup>i</sup>
2ip 2.0	66.27 <sup>d</sup>	0.74 <sup>ab</sup>	2.62 <sup>bcd</sup>
2iP 5.0	61.56 <sup>d</sup>	1.35 <sup>bc</sup>	3.63 <sup>de</sup>
2iP10.0	44.73 <sup>bc</sup>	1.76 <sup>bc</sup>	2.42 <sup>bc</sup>
TDZ 0.2	37.04 <sup>bc</sup>	1.73 <sup>bc</sup>	2.46 <sup>bc</sup>
TDZ 0.5	46.64 <sup>c</sup>	1.14 <sup>b</sup>	4.47 <sup>defg</sup>
TDZ 1.0	72.53 <sup>de</sup>	0.00 <sup>a</sup>	5.39 <sup>gh</sup>

PGR Plant growth regulator, BA 6-Benzyladenine, ZET Zeatin, KIN Kinetin, 2-iP 2-Isopentyladenine, TDZ Thidiazuron.

**Supplementary Table 3.** Effect of different auxins on root induction in PED cultivar.

PGR concentration (mg l <sup>-1</sup> )	Mean square values	
	% responding shoots <sup>1</sup>	Number of roots per shoot
0.5 IAA	38.95 <sup>b</sup>	4.39 <sup>bc</sup>
1.0 IAA	56.84 <sup>c</sup>	7.21 <sup>c</sup>
2.0 IAA	48.28 <sup>c</sup>	6.40 <sup>bc</sup>
0.5 IBA	100.00 <sup>e</sup>	13.18 <sup>d</sup>
1.0 IBA	86.70 <sup>d</sup>	6.65 <sup>bc</sup>
2.0 IBA	80.24 <sup>d</sup>	5.80 <sup>bc</sup>
0.5 NAA	33.33 <sup>b</sup>	3.81 <sup>bc</sup>
1.0 NAA	57.57 <sup>c</sup>	5.84 <sup>bc</sup>
2.0 NAA	55.23 <sup>c</sup>	3.20 <sup>ab</sup>
MS basal	11.03 <sup>a</sup>	3.20 <sup>ab</sup>

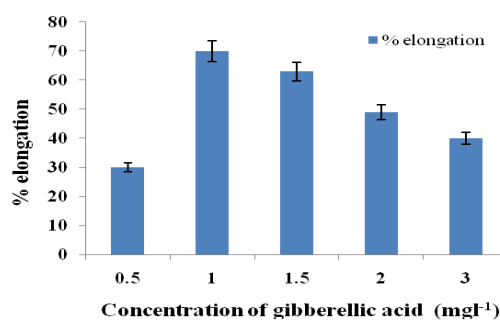
Means were compared by Duncan's Multiple Range Test (DMRT) and data having different letter within a column are significantly different (\*\*  $P < 0.05$ )

<sup>1</sup> Percentage of shoots which developed roots

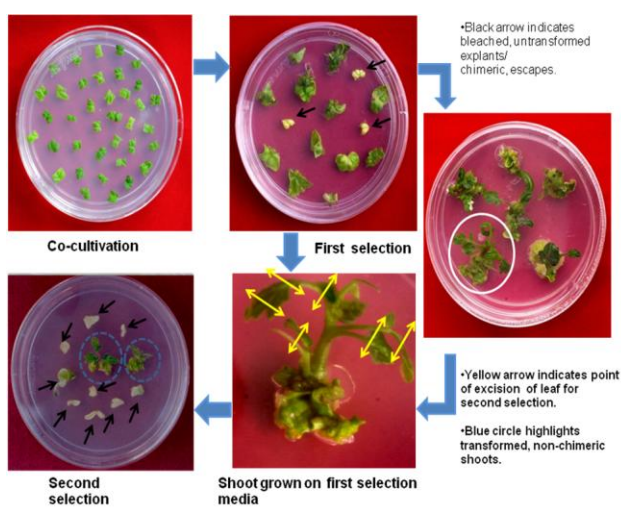
PGR plant growth regulator, IAA indole-3-acetic acid, IBA indole-3-butyric acid, NAA  $\alpha$ -naphthalene acetic acid, MS basal Murashige and Skoog's basal medium (1962).

**Supplementary Table 4.** Transformation efficiencies in different commercial cultivars with p35SGUS-INT vector using the modified leaf-disc procedure of tomato transformation.

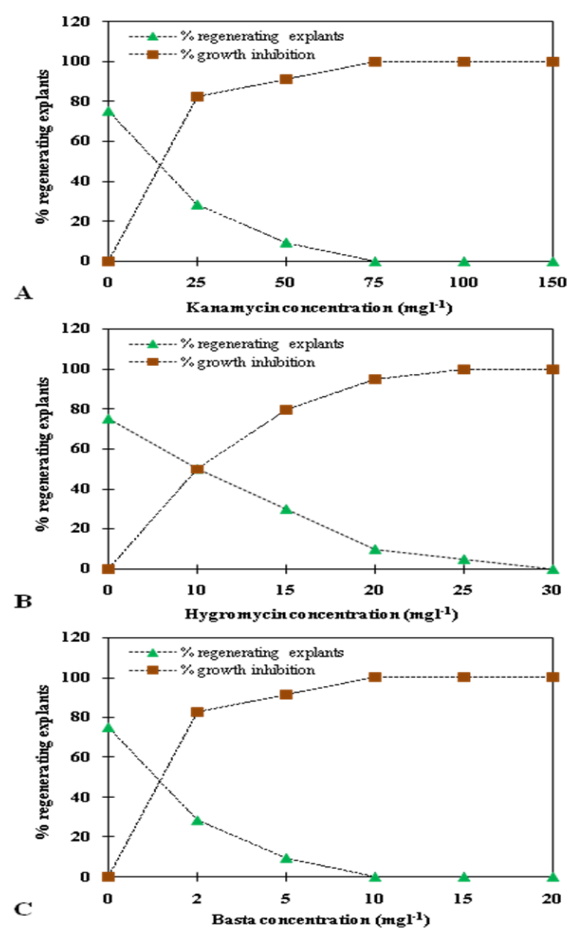
Tomato variety	Description/ characteristics	Responding explants (%)	Mean number of elongated shoots per explant	Transformation efficiency (%) (% ± SE)
PED	Early ripening cultivar, suitable for table as well as processing purpose	96.20	5.80	35.70 ± 1.2
Pusa120	Uniform ripening, heavy yielder and resistant to nematode.	47.11	4.05	27.15 ± 0.85
Pusa hybrid1	Fruit round, smooth, attractive, capable of fruit setting upto 28°C night temperature.	36.23	2.80	22.76 ± 1.6
S-22	Vigorous growth and excellent yield potential	57.96	4.83	27.83 ± 1.3
Pusa Ruby	Suitable for table as well as processing purpose	71.98	4.05	28.16 ± 0.98
Gaurav	Suitable for canning and long distance transportation	88.46	5.03	28.23 ± 0.2



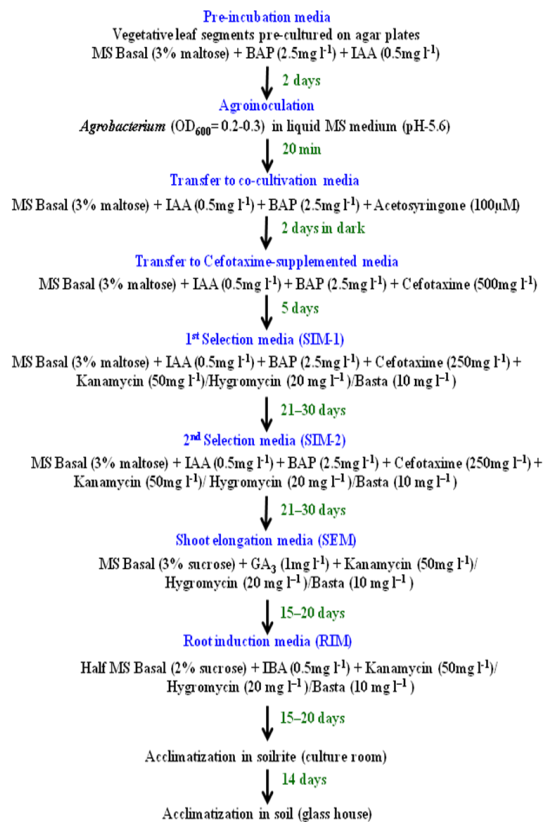
**Supplementary Fig. 1.** Effect of different concentrations of gibberellic acid on elongation of *in vitro* regenerated shoots of tomato (PED variety).



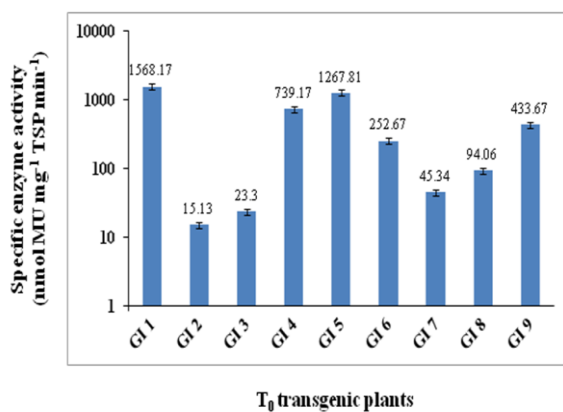
**Supplementary Fig. 3.** Technique of double selection for eliminating chimeras and escapes.



**Supplementary Fig. 2.** Antibiotic and herbicide sensitivity of leaf explants of tomato (*Solanum lycopersicum*). The optimized concentration of (A) kanamycin was found to be 50 mg l<sup>-1</sup>, (B) hygromycin was found to be 20 mg l<sup>-1</sup> and (C) basta 10 mg l<sup>-1</sup>, for selection of tomato.

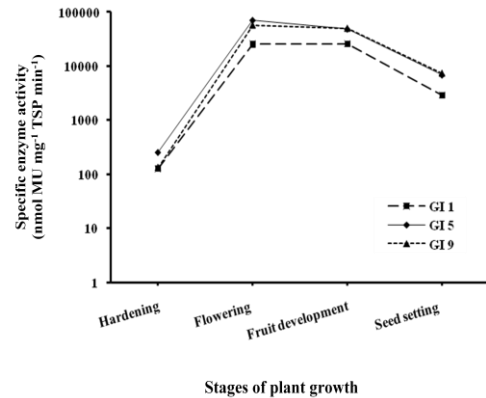


**Supplementary Fig. 4.** Schematic procedure for the development of transgenic tomato.



**Supplementary Fig. 5.** Fluorometric assay of GUS activity in independent T<sub>0</sub> transformed plants.

**S**  
**u**  
**p**  
**p**  
**l**  
**e**  
**m**  
**e**  
**n**  
**t**  
**a**



**ry Fig. 6.** Quantitative estimation of GUS expression in T<sub>0</sub> transgenic plants at different developmental stages.