BIOLOGIA PLANTARUM 54 (2): 344-348, 2010

BRIEF COMMUNICATION

Efficient production of transgenic tomatoes *via Agrobacterium*-mediated transformation

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Abstract

Cotyledonary leaves of 9-d-old tomato (*Lycopersicon esculentum* Mill.) were co-cultivated with *Agrobacterium tumefaciens* GV 3101 harboring binary vector pBI101 containing kanamycin resistance gene (*npt* II) as selection marker. Murashige and Skoog (MS) inorganic salts with Gamborg's B5 vitamins supplemented with optimized concentrations of zeatin riboside and indole-acetic acid resulted in enhanced regeneration efficiency. Under optimized conditions of plant regeneration, transformation frequency in cvs. Pusa Ruby, Pusa Uphar and DT-39 was greater than 37 %. Transformed shoots were selected on kanamycin medium and the presence of the transgene in the primary transformants was confirmed by PCR. Integration of the *npt* II gene in the tomato genome was further confirmed by Southern blot analysis. RT-PCR analysis using neomycin phospho-transferase (*npt II*) gene-specific primers confirmed the expression of the transgene in transgenic plants. Transformed plants were successfully transferred to phytotron, where these plants grew to maturity and produced flowers and fruits.

Additional key words: cotyledonary leaf explant, genetic transformation, *Lycopersicon esculentum*, zeatin riboside.

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Agrobacterium-mediated genetic transformation of tomato is still not a routine practice. Till date several methods for *in vitro* regeneration of tomato have been described and cotyledons are the choice explant for tomato plant regeneration as they are quickly established and possess a high morphogenetic potential. Regeneration *via* shoot morphogenesis has been reported using cotyledon as explant (McCormick *et al.* 1986, Van Roekel *et al*. 1993, Frary and Earle 1996, Ellul *et al.* 2003). In spite of numerous publications, much success has not been achieved towards improving the transformation efficiency (Hamza and Chupeau 1993, Hu and Phillips 2001, Cortina and Culianez-Macia 2004). In addition, most of the protocols have been optimized for only a few cultivars using either feeder layer, complex time consuming media formulation or successive subcultures layers (McCormick *et al*. 1986, Hamza and Chupeau 1993, Agharbaoui *et al*. 1995). Addition of phenolic compounds, acetosyringone to the co-cultivation has also been reported to increase the transformation frequency (Katia *et al*. 1993, Cortina and Culianez-Macia 2004). Moreover, different selection markers such as *bar* gene and phosphomannose isomerase (*pmi*) gene has also been used for increasing the transformation efficiency of tomato and other important vegetable crops (Cho *et al.* 2008, Bříza *et al.* 2008).

The present investigation describes the development of an efficient, reliable and simplified procedure of *Agrobacterium*-mediated transformation for tomato by avoiding the feeder layer, acetosyringone and eliminating frequent media changes, applicable to different cultivars of tomato as well as for the other *Solanaceous* species. This report could further aid in developing transgenic tomato with agronomically useful traits such as abiotic and biotic stress tolerance, improved shelf life and increased lycopene content.

Seeds of tomato (*Lycopersicon esculentum* Mill.) cvs. Pusa Ruby, Pusa Uphar and DT-39 were procured from Division of Horticulture, Indian Agricultural Research Institute, New Delhi, India. Seeds were surface

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Received 17 June 2008, *accepted* 10 January 2009.
Abbreviations: BA - benzyladenine; IAA - indole acetic acid; MS - Murashige and Skoog's medium; NPT II - neomycin phosphotransferase II; ZR - zeatin riboside.
Acknowledgement: The authors are grateful to the Department of Biotechnology, Government of India for supporting this research.
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sterilized in 4 % sodium hypochlorite solution for 8 min followed by three rinses in sterile distilled water. Surface-sterilized seeds (5 - 6 seeds per test tube) were germinated on half strength Murashige and Skoog (1962; MS) inorganic salts medium with B5 (Gamborg *et al*. 1968) vitamins (MSB5) and 30 g dm^{-3} sucrose. The pH of the medium was adjusted to 5.8 and solidified with 0.8 % agar (*HiMedia Laboratories*, Mumbai, India). Medium was sterilized by autoclaving at 121 ºC temperature for 20 min. Seedlings were grown at 25 ± 2 ºC with a photoperiod of 16-h (irradiance of 46 μmol m^{-2} s^{-1}). Cotyledonary leaf explant from 9-d-old *in vitro* grown seedlings were transversally cut into two segments and cultured on a pre-culture medium. Approximately 20 cotyledon explants were placed on the medium (in a 90-mm Petri dish) with the abaxial surface in contact with the medium. Throughout the study MSB5 medium alone or with different concentrations of benzyladenine (BA) or zeatin riboside (ZR; 0.5, 1.0, 1.5, 2.5 mg dm^{-3}) in combination with IAA (0.5 mg dm^{-3}) were tried for organogenic callus induction and shoot initiation. Regenerated shoots were further cultured on proliferation medium consisting of BA, ZR, IAA (0.5, 1 mg dm^{-3}) alone or in combination. For rooting, regenerated shoots were transferred onto MSB5 medium with IAA or NAA (0.1 - 0.5 mg dm^{-3}) and also on half strength MSB5 medium without any plant growth regulators. Prior to hardening, shoots with well-developed roots were transferred in half strength liquid MSB5 medium for 10 d before transfer to pots. Well rooted robust plants were then transferred to pots containing a mixture of peat, soil and vermiculite (2:1:1) moistened with sterile distilled water. Hardened plants were grown at 25 ± 2 ºC, 16-h photoperiod, irradiance of 200 μmol m^{-2} s^{-1}, and relative humidity of 62 %.

Agrobacterium tumefaciens GV 3101 harboring plant expression vector pBI101 (*Clontech*, Palo Alto, USA) with kanamycin resistant gene *npt* II as selection marker was used for tomato transformation. *A. tumefaciens* was grown in YEM medium (1.0 g dm^{-3} yeast extract, 10.0 g dm^{-3} mannitol, 1.0 g dm^{-3} NaCl, 0.2 g dm^{-3} $MgSO_4 \cdot 7\,H_2O$, 0.5 g dm^{-3} K_2HPO_4) containing the appropriate antibiotics to an absorbance (A_{600}) 0.6 to 0.8 at 28 ºC on rotary shaker (*Kuhner*, Basel, Switzerland) at 200 rpm prior to co-cultivation. Explants were excised and cultured onto the pre-culture media for 2 d on MSB5 medium supplemented with different concentrations of growth regulators. The pre-culture medium has the same composition as shoot regeneration medium without antibiotics. After 2 d, cotyledons were immersed in *Agrobacterium*-suspension containing *A. tumefaciens* strain diluted to one-tenth fold in liquid MSB5 medium. After immersion in bacterial suspension, the cotyledons were blotted dry and cultured on the same media for 2 d. The explants were then transferred to fresh selection medium consisting of different growth regulator combinations supplemented with kanamycin (50 mg dm^{-3}) and cefotaxime (250 mg dm^{-3}) (*HiMedia*) to control *Agrobacterium* growth. Every 2 weeks explants were sub-cultured onto the fresh selection medium. Kanamycin resistant regenerated plants were transferred to rooting medium supplemented with kanamycin (50 mg dm^{-3}) and cefotaxime (250 mg dm^{-3}).

At least three independent experiments were carried out with different culture media consisting of various combinations of plant growth regulators and at least 100 explants were tested each time. Analysis of variance was performed for the effect of various plant growth regulators on plant regeneration and transformation. Means were compared using the least significant difference test (LSD; $P < 0.05$).

Total genomic DNA was extracted from young expanding leaves of the primary transformants and the untransformed control using the DNeasy plant kit (*Qiagen*, Hilden, Germany) following the manufacturer's instructions. About 100 ng of genomic DNA was used to amplify the DNA fragment by PCR. The oligonucleotides 5'-CAATCGGCTGCTCTGATGCCG-3' and 5'-AGG CGATAGAAGGCGATGCGC-3' were used as primers amplifying a 700 bp fragment of the *npt* II gene. The PCR programme included DNA denaturation at 95 ºC for 3 min followed by 30 cycles of amplification (denaturation at 94 ºC for 30 s, primer annealing at 62 ºC for 30 s, and the extension at 72 ºC for 45 s) and finally 5 min at 72 ºC. The amplified product was visualized on 1 % agarose gel. Genomic DNA (10 μg) from transgenic and control plant (non-transformed tomato regenerant) were digested with *Hind*III and separated by electrophoresis in a 0.8 % agarose gel. DNA was transferred to a nylon membrane and hybridized with a *npt* II radioactive probe (Sambrook *et al***.** 1989). For the *npt* II probe an internal fragment of the gene was obtained by PCR amplification. Expression of the *npt* II gene in transgenic plants was analyzed by RT-PCR. Total RNA was extracted from leaves using RNeasy plant mini kit (*Qiagen*) following the manufacturer's instructions. For RT-PCR analysis, 500 ng of RNA free from any trace of DNA was first converted into cDNA and then amplified using *npt* II gene-specific primers by one step RT-PCR kit (*Qiagen*).

Callus induction as well as shoot initiation was observed from cotyledon explants cultured on various regeneration media. These media differed from each other regarding the difference in concentrations of growth regulators. In non-transformed cotyledon explants, gradual increase in regeneration frequency was observed with increasing concentrations of BA (1.0 - 2.5 mg dm^{-3}) in combination with IAA (0.5 mg dm^{-3}) in all the cultivars tested. The optimum regeneration frequency was observed on MSB5 medium supplemented with ZR (0.5 mg dm^{-3}) and IAA (0.5 mg dm^{-3}) (Table 1). Number of shoots produced per explant was also found to be highest when initiated shoots were transferred to proliferation medium with ZR (1.0 mg dm^{-3}). No regeneration response was observed from cotyledons cultured on MSB5 basal medium without growth regulators. Cotyledon explants co-cultivated with

Table 1. Effect of growth regulators on regeneration efficiency of three tomato cultivars. Means ± standard deviation (SD). Means followed by the same letters are not significantly different ($P < 0.05$) using LSD.

Shoot regeneration medium PGR [mg dm^{-3}]		Organogenic response [%]			Shoot proliferation medium PGR [mg dm^{-3}]		Number of shoots		
		Pusa Ruby	Pusa Uphar	DT-39			Pusa Ruby	Pusa Uphar	DT-39
BA	IAA				BA	IAA			
1.0	0.5	66.7	52.8	21.5	1.0	-	5.06 ± 1.10c	2.92 ± 1.10c	1.51 ± 0.10c
1.5	0.5	70.6	58.0	22.8	-	0.5	4.15 ± 0.11c	2.87 ± 0.96c	1.18 ± 0.10c
2.5	0.5	78.9	72.5	28.9	0.5	-	6.90 ± 0.98b	4.18 ± 0.11b	1.80 ± 0.11c
ZR	IAA				ZR	IAA			
0.5	0.5	90.9	82.2	55.5	1.0	-	8.70 ± 0.96a	6.90 ± 0.79a	3.12 ± 0.10a
1.0	0.5	88.0	78.0	46.0	0.5	0.5	7.01 ± 1.02b	4.55 ± 0.80b	2.50 ± 0.10b

Table 2. *Agrobacterium*-mediated transgenic shoot formation from three tomato cultivars on varying combinations of plant growth regulators. Transformation efficiency was calculated as the percentage of explants producing at least one transgenic plant. Means ± SD. Means followed by the same letters are not significantly different at $P < 0.05$ using LSD.

Selection medium PGR [mg dm^{-3}]		Transformation efficiency [%]			Shoot proliferation medium PGR [mg dm^{-3}]		Number of transformed shoots		
		Pusa Ruby	Pusa Uphar	DT-39			Pusa Ruby	Pusa Uphar	DT-39
BA	IAA				BA	IAA			
1.0	0.5	44.2	18.8	2.8	1.0	-	2.28 ± 0.11c	1.45 ± 0.11c	1.09 ± 0.10b
1.5	0.5	51.3	28.1	5.2	-	0.5	1.62 ± 0.19d	1.22 ± 0.09c	1.10 ± 0.11b
2.5	0.5	62.6	50.7	16.6	0.5	-	4.32 ± 0.12b	3.83 ± 0.09b	1.12 ± 0.09b
ZR	IAA				ZR	IAA			
0.5	0.5	71.6	67.5	37.1	1.0	-	7.60 ± 0.12a	5.12 ± 0.12a	2.06 ± 0.10a
1.0	0.5	68.5	63.1	28.9	0.5	0.5	4.81 ± 0.11b	3.16 ± 0.10b	1.60 ± 0.09b

Agrobacterium were cultured on different selection medium supplemented with kanamycin (50 mg dm^{-3}) and cefotaxime (250 mg dm^{-3}) (Table 2). As compared to control experiments (non-transformed tomato regenerants), a decrease in regeneration rate was observed in transformed explants. Maximum transformation efficiency up to 71.6 % was achieved when explants were cultured on selection medium containing ZR (0.5 mg dm^{-3}) + IAA (0.5 mg dm^{-3}) + kanamycin (50 mg dm^{-3}) + cefotaxime (250 mg dm^{-3}) producing 2 to 7 shoots per explant. Green kanamycin resistant callus along with shoot primordia was observed on the cut ends of cultured explants 2 to 3 weeks after transformation. After 4 to 8 weeks of shoot initiation, cotyledon explants were cut from the regenerating shoots and discarded. Enhanced shoot proliferation and elongation was observed after 10 weeks of culture on shoot proliferation medium supplemented with ZR (1.0 mg dm^{-3}) + kanamycin (50 mg dm^{-3}) + cefotaxime (250 mg dm^{-3}). Callus formation was also observed on all the concentrations of BA (1.0 - 2.5 mg dm^{-3}) tested in selection medium. BA supplemented medium enhanced the adventitious shoot buds formation; however, further shoot elongation could not be achieved. Leafy structures lacking defined shoot meristem were also developed frequently on increasing the concentration of BA (2.5 mg dm^{-3}). Regenerating shoots on proliferation medium were transferred to rooting medium with kanamycin (50 mg dm^{-3}). Half strength MSB5 medium without any growth regulator was found suitable for rooting of transformed shoots, where 80 % of shoots produced roots after 10 to 12 d. For better survival of transformed shoots, the plantlets were further transferred to liquid half-strength MSB5 medium supplemented with kanamycin (50 mg dm^{-3}) + cefotaxime (250 mg dm^{-3}), where hairy root formation was observed within 10 d. Transformed plants were hardened in small pots where 90 % survival rate was observed. Fully regenerated putative transgenic plants were transferred to phytotron, where these acclimatized plants grew to maturity and produced normal flowers and fruits. The transformants were observed to be morphologically normal and fertile. Putative transgenics transformed with *Agrobacterium* harboring kanamycin resistance gene (*npt* II) were obtained in all the three tomato cultivars. The transgenic plants were found to be PCR positive with *npt* II primers. PCR of nine such independently transformed plants is shown in Fig. 1*A*. An amplicon of 700 bp corresponding to the predicted size of gene fragment confirmed the transgene integration in the plant genome. No amplification was observed in the non-

transformed wild type plant (Fig. 1*A*). Transformants were further confirmed by Southern blot analysis (Fig. 1*B*) at T1 stage. Southern hybridization of genomic DNA from five randomly selected tomato plants (T1) using the *npt* II as probe showed single hybridization bands. This indicates npt *II* gene insertion as a single copy in the tomato genome. Southern positive transgenic plants were further analyzed by RT-PCR with *npt* II gene-specific primers for confirming the transgene expression. A 700 bp cDNA product corresponding to the *npt* II transcript confirmed the expression of the *npt* II gene in all the five transgenic plants. No amplification was observed in RNA samples isolated from the wild type plant. RT-PCR results of only five independent transgenic plants are shown in Fig. 1*C*. In the present study, optimal regeneration frequency was achieved with MSB5 basal medium supplemented with 0.5 mg dm^{-3} ZR in combination with 0.5 mg dm^{-3} IAA. The transformed shoots could be proliferated on selection medium supplemented with 1.0 mg dm^{-3} ZR. ZR has earlier been implicated in increased shoot regeneration in cultivar UCB2B (Van Roekel *et al*. 1993, Cortina and Culianez-Macia 2004). Contrary to our observations, Gunay and Rao (1980), reported combination of BA and IAA to be superior for shoot regeneration in tomato. Park *et al.* (2003) and Cortina and Culianez-Macia (2004), used NAA and BA in pre-culture medium; however, in the present investigation rhizogenesis was observed from explants cultured on NAA and BA combination (data not shown). MS medium is the most commonly used medium for tomato tissue culture (Chandel and Katiyar 2000, Park *et al*. 2001). We used the same medium but in combination with B5 vitamins, where concentration of thiamine HCl was ten-fold higher as compared to MS vitamins and observed increased cell-growth and shoot proliferation with reduced necrotic lesions in transformed tissues. These results were in confirmation with Raj *et al*. (2005). Previous reports indicated the use of feeder layers (petunia or tobacco) cell system for pre-culture culture (Hamza and Chupeau 1993, Agharbaoui *et al*. 1995), acetosyringone (Katia *et al*. 1993, Cortina and Culianez-Macia 2004), for co-cultivation and complex multiple media (Ellul *et al*. 2003, Park *et al*. 2003) to achieve higher transformation frequency. With the optimized conditions of plant regeneration and transformation, transgenic tomato plants in all the three cultivars (Pusa

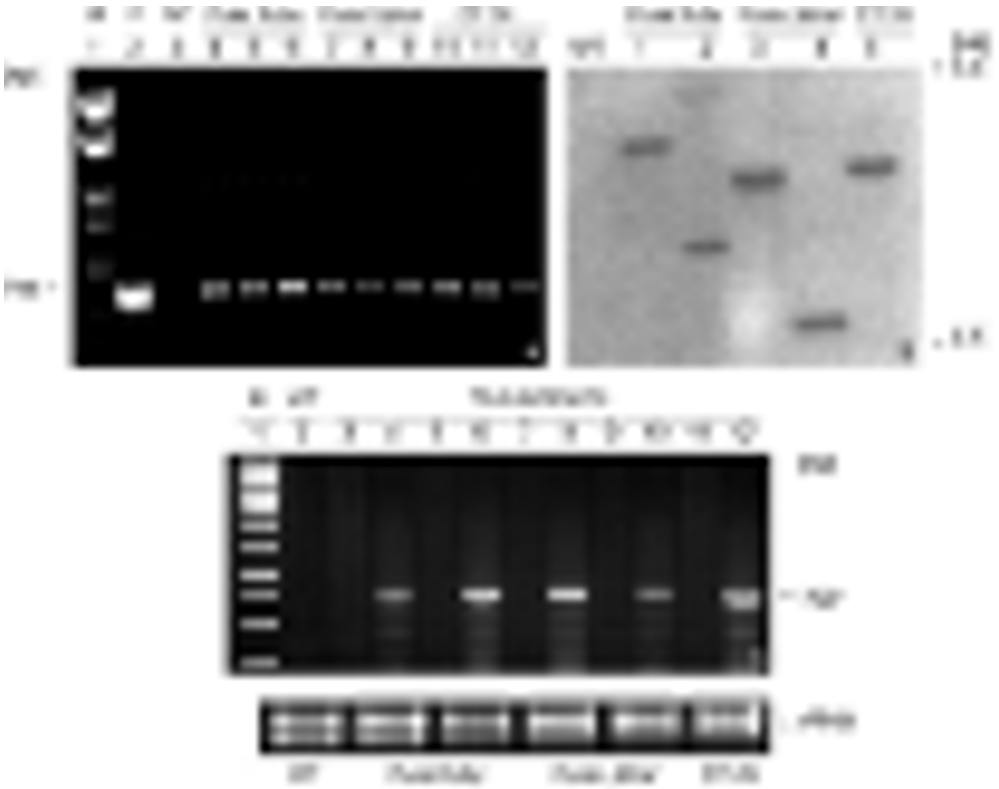

Fig. 1. PCR, Southern blot and RT-PCR analysis of transgenic tomatoes. *A* - PCR analysis of T_0 transformants of tomato using *npt* II gene-specific primers: *lane 1* - molecular mass marker (λ DNA cut with *Eco*RI + *Hind*III); *lane 2* - plasmid DNA as positive control; *lane 3* - wild type as negative control; *lanes 4 to 12* - putative transgenics. *B* - Southern-blot analysis of five randomly selected tomato plants (T1). Genomic DNA was digested with *Hind*III. *Lanes 1 to 5* - transformed tomato plants showing transgene integration and inheritance. *C* - RT-PCR analysis using the *npt* II gene-specific primers: *lane 1* - DNA size marker (1 kb ladder); *lane 2* - non-transformed wild type; *lanes 3, 5, 7, 9* and *11* - control without reverse transcriptase; *lanes 4, 6, 8, 10* and *12* - transgenics showing amplification of 700 bp fragment corresponding to the *npt* II transcript. Ethidium bromide-stained rRNA in the same gel is shown as a loading control.

Ruby, Pusa Uphar and DT-39) were obtained with transformation efficiency greater than 37 % about 10 weeks after commencing *in vitro* seed germination.

In conclusion, we have developed a simplified and improved genotype-independent regeneration protocol that is reproducible and reduces the time required to produce transformed shoots as it requires only two growth regulators and appropriate antibiotics for the production of *Agrobacterium*-mediated transgenic tomato for value added traits and functional genomics studies. Transgenics are being developed in our laboratory for abiotic stress tolerance and for delayed fruit ripening using this regeneration protocol.

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