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Research Article

STANDERDIZATION OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION PROTOCOL FOR TOMATO (*SOLANUM LYCOPERSICUM*)

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ABSTRACT

Agrobacterium mediated gene transformation method is considered as an effective method for transferring foreign genes into plants. Hence, this study was conducted to enhance foreign gene transformation efficiency of cultivars of tomato (*Solanum lycopersicum*) using Single colony *Agrobacterium* culture from freshly grown culture of LBA4404 containing T-*rep* gene construct in pCAMBIA 2301 was grown in 25 ml of Luria broth medium with appropriate antibiotics at 28^{0}C and 180 rpm for overnight. Infection was carried out in antibiotic free MS liquid medium and incubated for co-cultivation in dark for two days. Before the infection overnight grown *Agrobacterium* culture was re-suspended in antibiotic free Luria broth medium and allowed to grow to the log phase. Explants were then transferred to selection medium (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, 80mg/l kanamycin, 300mg/l cephotaxime pH 5.8). After 28 days of incubation at 25$\pm$2^{o}C in 16 hr photoperiod condition, explants responded with callus induction were transferred to fresh selection medium. The response of the callus induction on kanamycin selection was more or less similar in all sets of experiments. Most of the survived calluses on second selection medium were observed with shoot induction.

INTRODUCTION

Tomato is one of the popular vegetables in India. Yield and the quality of tomato are affected by many factors including pest and diseases, unfavorable weather conditions and post-harvest handling. The crop is highly subjected to post harvest losses during storage and transportation. Therefore crop improvement activities have been mainly directed towards the development of varieties with high yield, pest and disease tolerance and resistance to long term storage. Investigations on use of biotechnology for the improvement of Tomato have been carried out extensively in recent years. Gene-transformation is considered as the only method used to introduce genes with favorable characters. One of the most effective means of gene transfer into dicotyledonous plants has been reported as the *Agrobacterium tumefaciens* mediated transformation. It is well known fact that genes located within the border sequence of the *Agrobacterium* Ti plasmid are inserted into the genome of the host by random integration. Utilization of this mechanism for gene transfer requires both, susceptibility to infection by *Agrobacterium* and the ability to regenerate plants from individually transformed cells via tissue culture. A number of factors contribute to the overall efficiency of the *Agrobacterium* gene transformation. Up to now, the researchers have studied some factors affecting *Agrobacterium*-mediated transformation efficiency such as cocultivation duration, plant genotype, stage of explants, role of phenolic compounds, vector construct, *Agrobacterium* strain, bacterial density, infection temperature, and medium composition .Although a dicotyledonous plant secretes phenolic compound by itself, the addition of acetosyringone is found to be critical for stimulation of virulence gene action. This paper describes a successful approach to obtain regeneration of tomato plants from cotyledon explants and transformation of tomato by *Agrobacterium tumefaciens* method.

Agrobacterium mediated genetic transformation in tomato

Agrobacterium cells containing construct; pCAMBIA2301 with T-*rep* antisense gene along with NPTII gene as selectable marker gene (for antibiotic kanamycin as plant selection marker) &GUS gene as scorable marker gene was used for transformation of Tomato (var. TomD4) using protocol of McCormick (1991) with some modifications. Single colony *Agrobacterium tumefaciens* LBA4404 transformed with pCAMBIA2301 construct was grown in Luria broth liquid

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medium with rifamphicillin (25 µg/ml) and kanamycin (50 µg/ml) at 28°C for 18 hrs. With constant agitation at 180 rpm. 25ml of well grown culture was centrifuged at 4000 rpm at 4^0C for 5 min. pellet was then re-suspended in 25 ml of Luria broth medium without any antibiotic, with acetosyringone at the concentration of 200 uM. Culture was again incubated at 28°C for 18 hrs. With constant agitation at 180 rpm. till the OD reaches to ~ 0.8 (at $_{600\ nm}$). Finally before infection to the leaf explants the culture was diluted to 1:1 ratio using Murashige & Skoog liquid medium with 1% sucrose pH 5.8 before autoclaving.

Healthy seeds were sterilized with 0.1% HgCl2 for 2 minutes, followed by sterile distilled water wash for 6-7 times and placed on germination medium (1/2 MS medium) after blot drying on sterilized blotting paper. Seedlings were developed in 16 hr photoperiod at 25± 2^oC for one week after initial incubation in dark for 2-3 days. Cotyledonary leaves each were excised from 10 days old seedlings and explants were prepared by cutting all the sides of cotyledonary leaves. Placed the prepared leaf explants of size ~ 0.5 cm2 in adaxial position on the pre-culture medium (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 adjusted before autoclaving). Cultures were incubated at 25±2^oC in 16 hr photoperiod for two days before infection.

Infection was carried out in sterile glass beaker, two day pre-cultured leaf explants were removed for tissue culture medium safely without keeping any traces of phytoagar before infection. 50 leaf explants at a time were infected with the *Agrobacterium* culture prepared as described above. Cultures were left for 20 minutes for Agroinfection in dark with gentle shaking. Excess *Agobacterium* culture was removed. These co-cultivated explants were blot dried on sterile filter paper and then placed on co-cultivation medium (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 adjusted before autoclaving). Incubated the cultures in dark for 2 days at 25 ^{0}C.

The cotyledonary leaves were then transferred to (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 adjusted before autoclaving) 80mg/l kanamycin, 300mg/l cephotaxime was added to autoclaved medium and poured in sterilized glass plate. The cultures were incubated at 25°C under 16 hr photoperiod for 3 to 4 weeks, with sub culturing at every 15-20 days. Young shoots were then transferred to MS medium containing 3% sucrose, 0.1 mg/l Zeatin, 0.1 mg/l IAA and kanamycin at 50 mg/l for shoot elongation. The shoots so obtained were transferred to MS medium with 0.05 mg/ml of IBA for rooting. Cultures were incubated for 2 to 3 weeks and rooted plants were transferred to pots containing peat and hardened. Hardened plants were shifted gradually to green house for further establishment and analysis. The observations on plant regeneration after co-cultivation were recorded. The seedlings were transplanted and maintained in greenhouse for further analysis along with a set of control plants.

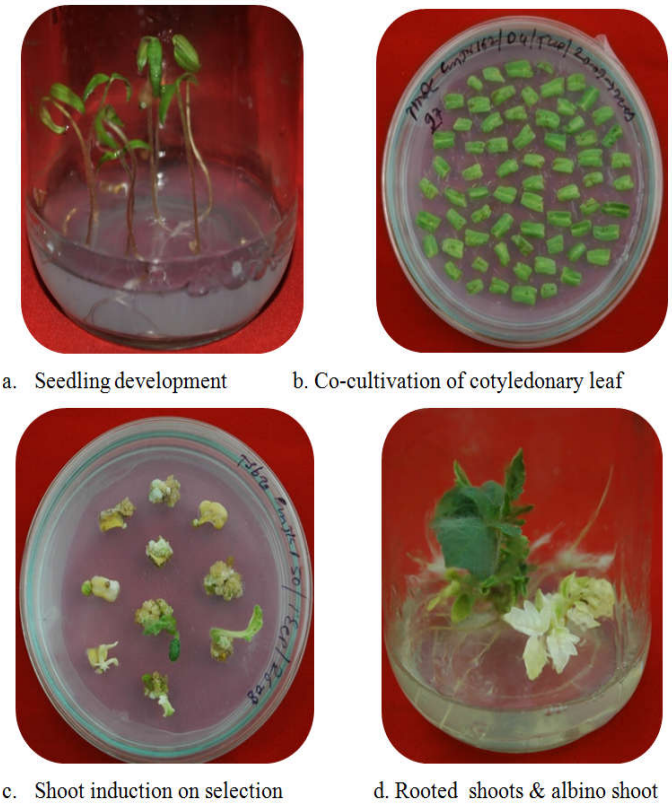
RESULTS AND DISCUSSION

Table Transgenic Tomato development summary

Experiment	No. of explants infected	Percent of explants responded on selection	Percent of callus induced shoots	No. of plantlets developed
Set 1	156	48	28	11
Set 2	151	42	33	8
Set 3	145	46	40	13
Set 4	147	40	35	17
Set 5	155	51	35	10
Set 6	150	39	32	7

Regenerated shoots from callus were harvested & transferred to selection free elongation medium (MS salts with B5 vitamins, 3% Sucrose and 300mg/l cephotaxime. pH 5.8). Well developed shoots transferred to half-strength MS medium with 300mg/l Cephotaxime pH 5.8 for rooting. After 15 days root initiation started & seventy three rooted plantlets transferred to primary hardening and kept in controlled condition green house with high humidity (< 90%). Well established plantelts were then transferred to soil in contained polyhouse as per DBT norms. Plantlets were grown till maturity and seeds were collected from self pollinated fruits. Leaf sample from these primary transformants were collected for histochemical GUS assay, genomic DNA isolation for PCR analysis to confirm the gene integration prior to maturity.

Figure: Steps during Agrobacterium mediated transgenic tomato development



a. Seedling development b. Co-cultivation of cotyledonary leaf

c. Shoot induction on selection d. Rooted shoots & albino shoot

e. Plantlets ready to harden

f. Hardening of plants

Histochemical GUS assay

Gene construct T-*rep* used in this study to develop transgenic tomato was cloned in pCAMBIA 2301 which has GUS reporter gene along with selection marker nptII gene. During transformation experiments kanamycin antibiotic was used as selectable marker and GUS as reporter gene to check the gene expression at different stages of transformation in laboratory. To confirm the expression of inserted gene in primary transgenic tomato plant was carried out using the leaf pieces of the well established plants from contained polyhouse. Among the plants tested thirty plants were showing prominent GUS expression, control check was used from non transgenic leaf tissue from the tomato seedling which was positive control in transformation experiments. These GUS expressing plants were further used to confirm the gene integration by polymerase chain reaction (PCR).

Transgenic leaf expressing GUS gene

Non transgenic control

CONCLUSION

Tomato was successfully transformed & regenerated using cotyledon explants cultured on MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 and this has been proved by GUS histochemical assay hence this protocol can be utilized for transferring gene of interest to Tomato system.

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