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Optimization of Regeneration and Transformation Protocol of Tomato (*Lycopersicon esculentum* L.) Variety Vybhav with GUS Gene

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Abstract

Conditions for regeneration and transformation of *Tomato (Solanum lycopersicum,* syn. *Lycopersicon esculentum* L.) variety Vybhav through organogenesis was optimized in this study. Two different explants namely, cotyledons and hypocotyls excised from mature seeds 15 days after germination. The explants were tested for regeneration potential on Murashige and Skoog (MS) medium enriched with varied levels of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). A medium composition comprising 2.5 mg/l BAP and 0.1 mg/l IAA showed 93.75 and 80% regeneration in cotyledons and hypocotyls explants respectively. Shoot elongation was faster on MS medium containing 3 mg/l BAP where as profuse rooting was achieved in 0.1 mg/liter IAA. The optimized protocol for cotyledonary explants was further tested for its adaptability using *Agrobacterium* having a gene construct of hygromycin phosphotransferase *(hytII)*for plant selection and β-glucuronidase (GUS) for histochemical screening of transformants. Putative transformants (T_0) were evaluated by GUS assays and polymerase chain reaction (PCR) for *hptII*. *Agrobacterium* infection of tissues for 15 minutes and co-cultivation for 2 days revealed the integration of the reporter gene.

Key words : Tomato, Regeneration, *Agrobacterium*, Transformation.

Development of genetically modified transgenic plants with ergonomically important traits is one of the most important applications of recombinant DNA technology. Recent advances in genetic engineering techniques, however, has provided us with relatively easy ways to transfer genes from diverse sources into a wide range of crop plants, which would have been otherwise impossible with conventional plant breeding practices. Many genetically altered transgenic varieties of important staple crops, incorporated with genes for resistance to herbicide, pests and diseases have been commercially released in the last decade, which showed a great potential to benefit farmers and consumers. Transformation by *Agrobacterium* remains as the primary choice to incorporate intact gene constructs with defined boundaries in dicot plants, owing to the greater susceptibility of dicotyledons to this bacterium. Regeneration of Tomato was successfully achieved by several workers from cotyledons, leaves, and hypocotyls (1). Similarly, several methods for genetic transformation of Tomato (*Lycopersicon esculentum* L.) via *Agrobacterium* have been described by several scientists (2, 3). Success of *Agrobacterium* mediated transformation in a particular plant species depends largely on their ability to regenerate on tissue culture medium. Hence, in this study, we attempted to optimize regeneration and transformation protocol for the tomato variety Vybhav using *Agrobacterium* strain LBA4404 containing a plasmid vector harbouring chimeric GUS reporter (β- glucuronidase) gene and hygromycin phosphotransferase II *(hptII)* gene.

Methods

Plant Materials

Seeds of tomato variety Vybhav were surface sterilized with 70% (vol/vol) ethanol for 10 sec, followed by 4% sodium hypochlorite for 5 min, and rinsed in sterile water. Seeds were germinated on MS (4) medium containing 3% sucrose in a growth room at 25 ^{0}C under 16-h photoperiod with a light intensity of 65 mol/m^{2}s.

Preparation of Explants

All subsequent *in vitro* culture steps were conducted in a chamber with the growth conditions of

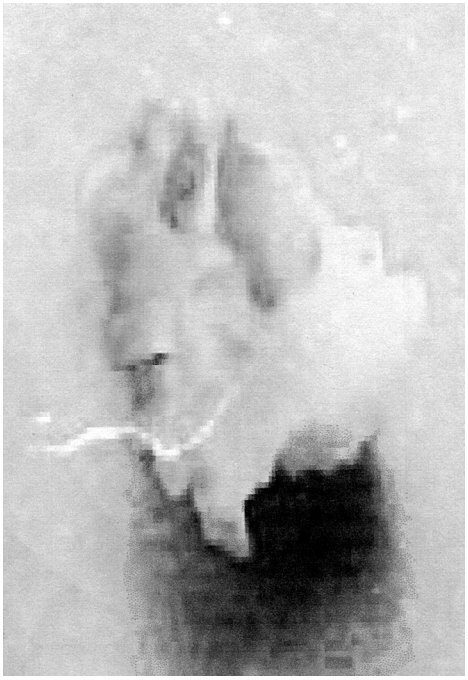

Figure 1. Histochemical GUS expression in the putative transgenic tomato callus.

16 h light, 25C and 70% RH. Seedlings were used 15 days after germination (5). Cotyledons were cut transversely with a sterilized scalpel into two halves of 3 × 2 mm size. Similarly hypocotyls were obtained from 15 days old *In vitro* grown plants. Excised cotyledons and hypocotyl explants were cultured on MS basal medium supplemented with different combinations of BAP and IAA. The number of calli produced after 30 days were recorded and then the regenerants were transferred onto a shooting medium for shoot elongation. The shoot elongation medium contained MS basal salt and different concentrations (0.1, 0.2, 0.3, 1.0, 2.0 and 3.0 mg/liter) of BAP. After shoot elongation, they were transferred to MS rooting medium containing 0.1-0.3 mg/liter IAA. Rooted plants were transferred to sterile peat substrate and kept in culture room for acclimatization and then maintained in a greenhouse.

Agrobacterium Strain and Plasmid

Agrobacterium strain LBA4404 harbouring the binary vector pABC [which carried hygromycin phosphotransferase *(hptII)* for plant selection and β-glucuronidase *(gusA)* for histochemical identification of transformants were used for transformation of Tomato.

Transformation of Tomato

Agrobacterium was grown overnight in YEP medium having 50 mg/l kanamycin. The bacterial suspension was centrifuged at 3000 xg for 5 min, resuspended in liquid MS medium and the OD was adjusted to 0.4-0.8 OD_{600} before infecting the explants. The explants were immersed in *Agrobacterium* suspension for 15 minutes. Infected explants were blotted dry on sterile filter paper and cocultivated for 2 days on fresh MS medium. The explants were then transferred onto MS medium containing BAP, IAA, 400 mg/l cefotaxime and 40 mg/l hygromycin. After 3 weeks, elongated shoots emerging from the cut surfaces were excised and placed on a medium (MS +IAA) for rooting. Transformation frequency was expressed as the percentage of inoculated explants producing transgenic plants. A transformant was defined as a shoot regenerated on medium containing 50 mg/liter hygromycin.

Histochemical GUS Assay

In situ detection of GUS activity in plant tissues was done according to Jefferson et al. (6). Explants were washed for 30 min in 50 mM phosphate buffer (pH 7.0) and immersed for 10 min in a fixation solution (0.3% formaldehyde, 10 mM MES, 0.3 M mannitol). The samples were kept immersed in 1 mM X-Gluc (5-bromo-4-choloro-3-indoly-β-D-glucuronic acid) staining solution and incubated overnight at 37 C for the color development.

PCR Assay

The genomic DNA of tomato was extracted from young leaves of putative transgenic plants according to Sambrook et al. (7) and used in polymerase chain reaction (PCR). Amplification of *hpt*II gene with the primer pair *hpt*F : 5'-AAAAAGCCTGAACTCACCGC-3' and hptR : 5'-ACTTCTACACAGGCATCGGT-3' was done by em-

Table 1. Regeneration efficiency of cotyledonary and hypocotyl explants of tomato var Vybhav at different combinations of BAP and IAA in MS medium. The data are average of four replications. *Significance at 5% level.

Hormones (mg/l)		Cotyledonary explants		Hypocotyls explants	
BAP	IAA	Number of explants regenerated	Percent regeneration	Number of explants regenerated	Percent regeneration
0.0	0.0	0.75	12.50	1.00	10.00
2.0	0.1	8.50	85.00	7.25	72.50
3.0	0.1	9.00	90.00	7.75	77.50
4.0	0.1	2.00	20.00	1.75	17.50
5.0	0.1	1.50	15.00	1.25	12.50
2.0	0.2	5.75	57.50	5.00	50.00
2.0	0.3	3.50	27.50	4.50	45.00
2.0	0.4	4.50	45.00	4.00	40.00
2.0	0.5	7.25	72.50	5.25	52.50
2.5	0.1	7.50	93.75	8.00	80.00
F test		*		*	
$CD_{0.05}$		1.12		1.14	
SE		0.38		0.39	

ploying standard protocol (7). The PCR reactions were carried out in a total volume of 25 ml, containing 1µl DNA, 0.7 µl of each primer, 0.7 µl of 2.5mM dNTP mix, 1.0 µl of *Taq DNA polymerase* (3 units/µl) and 2.5 µl of *Taq* DNA polymerase assay buffer with 15 mM $MgCl_2$. DNA samples from plasmid DNA as positive control. DNA from transformed plant samples and DNA from control plant samples as negative control were used for PCR. The temperature profile used in PCR was as follows : initial denaturation of DNA at 94 C for 5 min, followed by 35 cycles of 1 min denaturation at 94 C, 1 min annealing at 55 C, 1.5 min elongation at 72 ^{0}C and a final extension step at 72 C for 6 min. Amplification products were analyzed by electrophoresis on 1% agarose gel.

Statistical Analysis

Each treatment of an experiment was conducted with at least 10 explants and each experiments was repeated four times and the mean value was calculated. Student *F* test was used to compare the treatment means for significance.

Results and Discussion

Tomato is an economically important crop in many countries, including India. The integrated ap-

Table 2. Effect of BAP on shoot elongation of cotyledonary and hypocotyl explants of tomato. *Mean value of four replications. **Significant.

Treatments (mg/l)	Cotyledonary explants		Hypocotyls explants	
	Number of shoots elongated*	Per cent	Number of shoots elongated*	Per cent
MS + BAP (0.1)	1.25	15.62	0.75	09.37
MS + BAP (0.2)	3.25	40.62	2.50	31.25
MS + BAP (0.3)	2.75	34.37	2.00	25.00
MS + BAP (1.0)	4.00	50.00	2.75	34.37
MS + BAP (2.0)	4.75	59.37	3.25	40.62
MS + BAP (3.0)	6.50	81.25	6.00	75.00
F test	**		**	
$CD_{0.05}$	0.99		1.10	
SE	0.34		0.36	

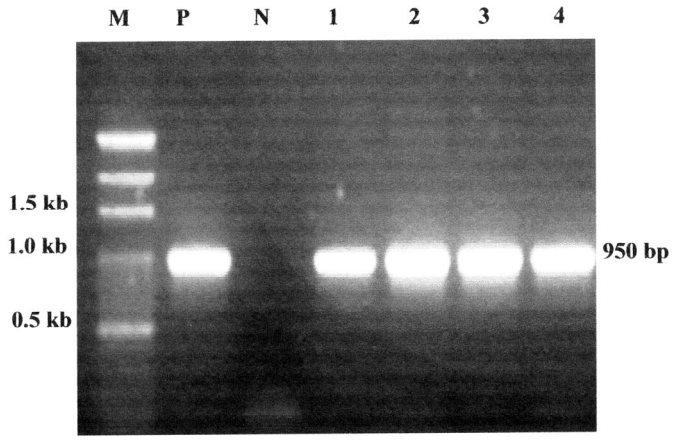

Figure 2. PCR amplification of *hpt*II gene in the putative (T0) Tomato tranformants. M-100bp marker ; P-positive control (pABC) ; N- nontransformed Tomato ; 1-4 : putative transfenic Tomato transformed with pABC through *Agrobacterium.*

proaches of genetic engineering of Tomato have a great value because of its commercial importance. Generation of genetically modified transgenic plants with a range of added trait is one of the most important applications of recombinant DNA technology. *Agrobacterium* mediated transformation offers an effective approach for genetic manipulation of crop plants. Tomato is relatively susceptible to *Agrobacterium* infection, and thereby become amenable to current transformation methods. For the successful application of genetic modification methods to improve agronomic traits, establishment of a reliable and reproducible regeneration and transformation procedure is critical (8).

Plant Regeneration

A good callus growth was seen in cotyledon explants after 4 weeks. In hypocotyl explants, the callus growth was visible on week 3 and good callus was obtained after week 5. The emergence of shoots from cotyledonary explants derived callus was started after 30 days and in hypocotyls the callus was observed on 35 days after explanting. Percent regeneration of callus originated from cotyledonary explants ranged from 12.5 to 90%, while in hypocotyls explants it ranged from 10 to 80% (Table 1). In cotyledonary system, a treatment containing 3.0 mg/l BAP and 0.1 mg/l IAA induced highest percentage (90%) of regeneration followed by 2.0 mg/l of BAP and 0.1 mg/l of IAA (85%). However, maximum callus formation was observed (93.75%) in the MS medium composition having 2.5 mg/l of BAP and 0.1 mg/l of IAA. The treatment combination of MS, 5.0 mg/l BAP and 0.1 mg/l of IAA recorded the lowest percentage of regeneration (12.5%) among the different hormonal combinations tested. It has been known that the potential for callus induction and regeneration in tomato tissue culture depends upon a number of factors such as genotype of target crop, the type of explants used, the composition and the concentration of the minerals, organic constituents and plant growth regulators in the tissue culture medium. Previous studies by several workers with cotyledons and hypocotyls showed 80-90% regeneration in tomato on medium containing BAP and IAA at different concentrations (9—11).

After the shoot initiation was achieved, the regenerated calli were transferred into shoot elongation medium (MS + BAP). Shoot elongation from the callus emerged from cotyledons was highest (81.25%) on MS medium containing 3 mg/l BAP followed by 2 mg/liter BAP (59.37%). The shoot elongation from callus of hypocotyls was 75% and 40.62% on 3 and 2 mg/liter of BAP respectively. After 45 days of incubation, the shoots attained the height of 3-4 cm. They were transferred to MS basal medium with different concentrations (0.1–0.3 mg/liter) of IAA for rooting. The MS medium containing 0.1 mg/liter of IAA has promoted better rooting. Results of these experiments identified cotyledonary explants as better choice for transformation because of its elevated levels of regenerative capabilities. Plant growth regulators play

a major role in plant tissue culture. A proper combination of auxin and cytokinin is critical for efficient regeneration. In this study, we observed adventitious root formation in low auxin (IAA) concentrations, while at higher concentration root formation failed and callus phase become dominant. Chawla (12) found that higher concentration of cytokinins induced shoot formation (Table 2).

The regenerated calli were transferred onto different concentrations of BAP for shoot elongation. Better shoot elongation was obtained on media containing 3 mg/liter of BAP, with a rate of 81.25 and 75% for cotyledonary and hypocotyl explants respectively. Similar results were observed by Chaudary et al. (13). They achieved higher shoot regeneration of calli derived from hypocotyls of the tomato cv Nagina on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/liter IAA and the mean number of shoots regenerated per explant was also high on the same medium. Rooting of *in vitro* formed shoots was achieved in media supplemented with IAA. The results of this study are in agrement with the report of Venkatesha (14).

To make use of this established regeneration protocol for *Agrobacterium*-mediated transformation, catyledonary explants were cocultivated with LBA 4404 and the transformants were selected on hygromycin. In this study, the cotyledonary explants were precultued for 2 days before being infected with *Agrobacterium.* Preculturing of the excised tissues for 2 days before co-cultivation is highly encouraged as wounding helps in the accumulation of phenolic substances during preculturing, which in turn induce the *vir* operons located in *Agrobacterium* Ti plasmid, thus facilitating T-DNA transfer (15).

Agrobacterium-Mediated Transformation

The cotyledonary explants were infected with 0.4, 0.5, 0.6, 0.7 and 0.8 OD cultures for 15 min. After the *Agrobacterium* infection, co-cultivation was continued for 48 h in dark. The *A. tumefaciens* culture having 0.4—0.5 OD_{600} showed 32.49% of cotyledonary explants survival. Further, the cotyledonary explants were transferred to the regeneration medium containg 2.5 mg/liter of BAP and 0.1 mg/liter of IAA after 48 hours of co-cultivation. The over growth of *Agrobacterium* during regeneration, was controlled by using the antibiotic cefotoxime (400 mg/liter) and regenerated shoots were placed on MS medium supplemented with 3 mg/l BAP and 40 mg/liter hygromycin for elongation. These putatively transformed shoots after elongation were rooted on MS basal medium comprising 0.1 mg/liter of IAA.

Standardization of optimum co-cultivation time and Agrobacterium concentration are essential for efficient gene transfer. In this study, inoculation of explants for 15 min with *Agrobacterium* culture of 0.4-0.5 OD_{600} followed by 2 days of co-cultivation was found suitable for effective regeneration of transformants. Co-cultivation for more than 2 days has resulted in overgrowth of bacterium and death of the tissues. Park et al. (15) recommended the co-cultivation period of 2 days for better results. After co-cultivation, the explants were transferred to the regeneration medium containing cefotaxime to check the bacterial growth on the explants. A cefotaxime concentration ranging from 200 to 500 mg/l has been used for checking *Agrobacterium* overgrowth in tomato (16). In the present study, culturing of explants on 400 mg/l cefotaxime effectively blocked the overgrowth of the bacterium.

Molecular and Biochemical Analysis of Putative (T_0) Transformants

Histochemical GUS assay was performed by overnight incubation of putatively transformed tomato callus with X-Glue staining solution. Intensive blue coloration was seen on callus where X-Gluc was catabolized by β-glucuronidase (GUS) activity (Fig. 1). However, GUS expression was not visualized in the control tissues. The genomic DNA from the leaves 2-months old putative transgenic plants were extracted and amplified with *hpt*II primers. A PCR amplicon of 950 bp was seen in the DNA of transgenic plants and the fragment was absent in nontransformed plants (Fig. 2), suggesting that the stable integration of T-DNA with intact *hpt*II and GUS genes into tomato genome. The transgenic plants were also confirmed for the integration of *hpt*II gene by PCR. Thus, molecular and biochemical analyses revealed the insertion of intact T-DNA into tomato genome.

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