Plant Tissue Cult. & Biotech. **19**(1): 101-111, 2009 (June)

PTC&B

In vitro Regeneration and *Agrobacterium*-mediated Genetic Transformation of Tomato (*Lycopersicon esculentum* Mill.)

R.H. Sarker, Khaleda Islam and M.I. Hoque

Department of Botany, University of Dhaka, Dhaka 1000, Bangladesh

Key words: In vitro regeneration, Transformation, Tomato

Abstract

Agrobacterium-mediated genetic transformation system has been developed for two tomato (*Lycopersicon esculentum* Mill.) varieties, namely Pusa Ruby (PR) and BARI Tomato-3 (BT-3). Prior to the establishment of transformation protocol cotyledonary leaf explants from the two varieties were cultured to obtain genotype independent *in vitro* regeneration. Healthy multiple shoot regeneration was obtained from the cut ends of cotyledonary leaf segments for both the varieties on MS containing 1.0 mg/l BAP and 0.1 mg/l IAA. The maximum root induction from the regenerated shoots was achieved on half the strength of MS medium supplemented with 0.2 mg/l IAA. The *in vitro* grown plantlets were successfully transplanted into soil where they flowered and produced fruits identical to those developed by control plants. Transformation ability of cotyledonary leaf explants was tested with *Agrobacterium tumefaciens* strain LBA4404 harboring binary plasmid pBI121, containing GUS and *npt II* genes. Transformed cotyledonary leaf explants were found to produce multiple shoots on MS containing 1.0 mg/l BAP and 0.1 mg/l IAA. Selection of the transformed shoots was carried out by gradually increasing the concentration of kanamycin to 200 mg/l since kanamycin resistant gene was used for transformation experiments. Shoots that survived under selection pressure were subjected to rooting. Transformed rooted plantlets were transferred to soil. Stable expression of GUS gene was detected in the various tissues from putatively transformed plantlets using GUS histochemical assay.

Introduction

Tomato (2n = 2x = 24) is one of the most widely produced and popular vegetables of the world. It ranks second to potato in many countries and tops the list in cane-vegetables (Tiwari and Choudhury 1986). Tomato is known as a protective food because of its special nutritive value. Tomatoes are particularly very popular since they can be eaten fresh or in a multiple of processed forms. The intensive cultivation of tomatoes has led to a significant increase in

the farmer's income in many developing countries. A multiple of complex type of pests and diseases as well as post harvest losses threaten the stability of production of tomato. In total, there are more than 200 pathogens that infect the tomato crop and often are the limiting factor in tomato production (Jones et al. 1997). These are: viruses, viroids, fungi, bacteria, nematodes, parasitic weeds, pests and insects. The most devastating among them begomoviruses known by their generic names 'tomato leaf curl virus' and 'tomato yellow leaf curl virus'. So far, 18 distinct begomoviruses associated with tomato leaf curl disease (ToLCVD) and 11 associated with tomato yellow leaf curl disease (TYLCVD) have been identified (Varma and Malathi 2003). These viruses are transmitted by white flies (*Bemisia tabaci* Genn.). Tomato production is very much pesticide intensive and the farmers usually spray against whitefly almost everyday. Moreover, accurate information is lacking about the action of pesticides and the farmers often have inadequate knowledge about the cause of diseases and the effect of various pesticides. An extensive use of these costly chemicals creates notable environmental and social problem in most developing countries (Rojas et al. 2003). Fungicides and pesticides residues enter the human food chain and may cause serious illnesses. Under these circumstances it is a better alternative to use disease and insect-pest resistant varieties instead of chemical control. To overcome the constraints of tomato production various breeding and biotechnological techniques are being applied in various parts of the world (Ling et al. 1998, Oktem et al. 1999 and Park et al. 2003). With the advent of genetic transformation it has been possible to transfer disease and pest resistant genes beyond the boundaries of species. The most common method for transfer of desired foreign gene(s) into dicotyledonous plants is *Agrobacterium*-mediated genetic transformation. For successful genetic transformation a suitable reproducible regeneration protocol is essential. The present investigation has been carried out for the development of a protocol for *Agrobacterium*-mediated genetic transformation of locally available tomato varieties so that the desired genes can be introduced for the development of disease as well as pest resistant varieties.

Materials and Methods

Two varieties of tomato (*Lycopersicon esculentum* Mill.), namely BARI Tomato-3 and Pusa Ruby were used in this study. Seeds of the variety BARI Tomato-3 were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur and the variety Pusa Ruby were collected from the local market provided by East West Seed (Bangladesh) Ltd.

For the preparation of explants the seeds were washed with 70% (v/v) ethanol for 1 min and surface sterilized in the laminar flow with 2.5% (v/v) sodium hypochlorite solution containing 0.1% Tween-80 for 20 - 30 min. The seeds were then washed five times with sterilized distilled water. To facilitate germination, seeds were immersed in sterile distilled water and were shaken on a shaker (100 rpm) for 36 - 48 h. These were then cultured on agar solidified MS with 3% sucrose. Cotyledonary leaves were collected from 8-10 days old *in vitro* grown seedlings. Each cotyledonary leaf was transversely cut into two - three segments and each segment was used as explant.

For induction and production of multiple shoots from cotyledonary leaf explants, MS supplemented with different concentrations and combinations of BAP, IAA and Kn were used. For root formation from the cut ends of regenerated excised shoots, full and half strengths of MS with different combinations and concentrations of IBA, IAA and NAA were used. All media combinations contained 3% sucrose solidified with a 0.8% agar and with a pH of 5.8, adjusted before autoclaving. Cultures were maintained under a regime of 16 h photoperiod at 25 ± 2ºC. Following the development of sufficient roots, plantlets were transferred to small plastic pots containing sterilized soil. These plantlets were acclimated and then transferred to the field and maintained there till flowering and fruiting.

Agrobacterium tumefaciens strain LBA4404 with the binary plasmid pBI121 was used for transformation. It contains a scoreable reporter gene GUS and and a selectable marker gene *nptII* encoding the enzyme neomycin phosphotransferase conferring kanamycin resistance (Herrera- Estrella et al. 1983). Fifty ml of liquid YMB (Hooykaas 1988) containing 50 mg/l kanamycin were inoculated with *Agrobacterium* from a fresh bacterial plate and grown at 200 rpm on a rotary shaker at 28ºC for 16 h. The culture was subsequently spun at 5000 rpm at 20ºC for 10 min in a centrifuge and the pellet resuspended in 10 ml liquid MS maintaining the optical density between 0.7 and 9.0.

The explants were prepared with a scalpel keeping the cotyledonary leaves submerged in the *Agrobacterium* suspension. The cut explants were incubated in the *Agrobacterium* suspension in a small Petri plate for an additional period of 30 min. They were then blotted dry on a sterilized Whatman filter paper and co-cultured in Petri plates on MS with 1.0 mg/l BAP and 0.1 mg/l IAA (best regeneration medium selected during regeneration experiments) for three days in the dark. Following co-culture the explants were washed several times in liquid MS with gentle shaking until no opaque suspension was seen. The infected explants were finally washed with 300 mg/l ticarcillin (Duchefa, Netherlands) dried with a sterile Whatman filter paper and placed on the regeneration medium with 50 mg/l combactum (Pfizer, Germany) and 50 mg/l

ticarcillin to control the over growth of bacteria. The infected explants were then placed in the growth room for regeneration under 16/18 hours light/dark cycle at 25 ± 2ºC.

To eliminate non-transformed tissues, the regenerating explants were sub-cultured on a fresh regeneration medium initially with 50 mg/l kanamycin after three weeks. The concentrations of selection antibiotic was increased with each sub-culture at 14 days intervals up to 200 mg/l kanamycin. During each subculture the dead and deep brown tissues were discarded and green shoots and shoot buds were sub-cultured on fresh medium containing the next higher concentration of kanamycin.

Transformation ability of explants was monitored by GUS histochemical assay (Jefferson et al. 1987) by submerging them in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl α-D-glucuronide) and incubating them at 37ºC for two - three days. They were then washed in 70% alcohol and scored for GUS expression. Shoots developing from uninfected tomato explants were used as negative control and transgenic tobacco tissues maintained in the laboratory were used as positive control. X-gluc was prepared by dissolving it in 100 µl dimethylformamide and made up to a final concentration of 10 mg/ml with 50 mM phosphate buffer at pH 7.0. Cultured explants were subjected to transient GUS assay after antibiotic wash (mentioned above). Tissues and shoots under selection pressure of around two months were monitored for stable GUS expression. Histochemical assays were carried out to observe the expression of GUS gene inside the explant tissues as well as in the different parts of putatively transformed plantlets.

Results and Discussions

Responses of all the different combinations and concentrations of growth regulators supplemented with MS for the induction of multiple shoots from cotyledonary leaf explants of both varieties are presented in Table 1. It was observed that BAP had positive effect towards multiple shoot regeneration. These results are in agreement with that of the findings of Oktem et al. (1999) and Fariduddin et al. (2004). However, kinetin (0.5 - 5.0 mg/l) supplemented MS did not show any response towards multiple shoot regeneration. In contrast to present findings Kartha et al. (1976) and Sheeja et al. (2004) were able to regenerate plants using moderate (2.0 mg/l) concentration of Kn using hypocotyle explants. The use of different explants in the present investigation may be the reasons for not achieving multiple shoot regeneration on Kn supplemented MS. Zeatin is another cytokinin reported to be used in *in vitro* regeneration and transformation of tomato (Ichimura and Oda 1995, Costa et al. 2000). BAP is commonly available and less costly cytokinin than zeatin and

Table 1. Effects of various combinations of BAP, Kn, and IAA in MS medium on regeneration and proliferation of multiple shoots from cotyledonary leaf segments of tomato var. Pusha Rubi (PR) and BARI tomato-3(BT-3).

Hormonal conc. (mg/l)			Variety	No. of explants inoculated	% of explants responded	Days for initiation of regeneration	Average No. of shoots/explant after 60 days of culture
BAP	Kn	IAA					
-	-	-	PR	50	00	-	-
			BT-3	50	00	-	-
0.5	-	-	PR	50	88.0	14 - 20	1.89
			BT-3	50	75.0	16 - 22	1.54
1	-	-	PR	50	92.0	14 - 22	2.96
			BT-3	50	90.0	14 - 22	2.59
2	-	-	PR	50	94.0	14 - 18	4.34
			BT-3	50	96.0	14 - 18	4.02
5	-	-	PR	50	90.0	12 - 15	4.56
			BT-3	50	92.0	14 - 18	4.23
-	0.5	-	PR	50	0	-	-
			BT-3	50	0	-	-
-	2	-	PR	50	4	18 - 22	0.04
			BT-3	50	3	20 - 22	0.03
-	5	-	PR	50	12	18 - 22	0.12
			BT-3	50	10	20 - 22	0.10
0.5	0.5	-	PR	50	90	15 - 20	1.90
			BT-3	50	87	16 - 22	1.32
1	0.5	-	PR	50	92	15 - 20	2.94
			BT-3	50	88.0	15 - 20	2.26
1	1	-	PR	50	94.0	14 - 18	3.15
			BT-3	50	92.0	15 - 20	2.34
2.0	0.5	-	PR	50	92.0	14 - 18	4.53
			BT-3	50	92	14 - 18	4.05
2.0	1.0	-	PR	50	94.0	14 - 18	4.59
			BT-3	50	92	14 - 18	4.12
0.5	-	0.1	PR	50	90	15 - 20	2.53
			BT-3	50		15 - 20	1.98
1.0	-	0.1	PR	50	96	14 - 18	4.92
			BT-3	50	88	14 - 18	4.25
2.0	-	0.1	PR	50	96	14 - 18	4.95
			BT-3	50	90	14 - 18	4.28
1.0	0.5	0.1	PR	50	96	14 - 18	4.91
			BT-3	50	92	14 - 18	4.27
1.0	1.0	0.1	PR	50	94	14 - 18	4.94
			BT-3	50	89	14 - 18	4.25
2.0	0.5	0.1	PR	50	92	14 - 18	4.93
			BT-3	50	92	14 - 18	4.29
2.0	1.0	0.1	PR	50	94	14 - 18	4.96
			BT-3	50	90	14 - 18	4.29

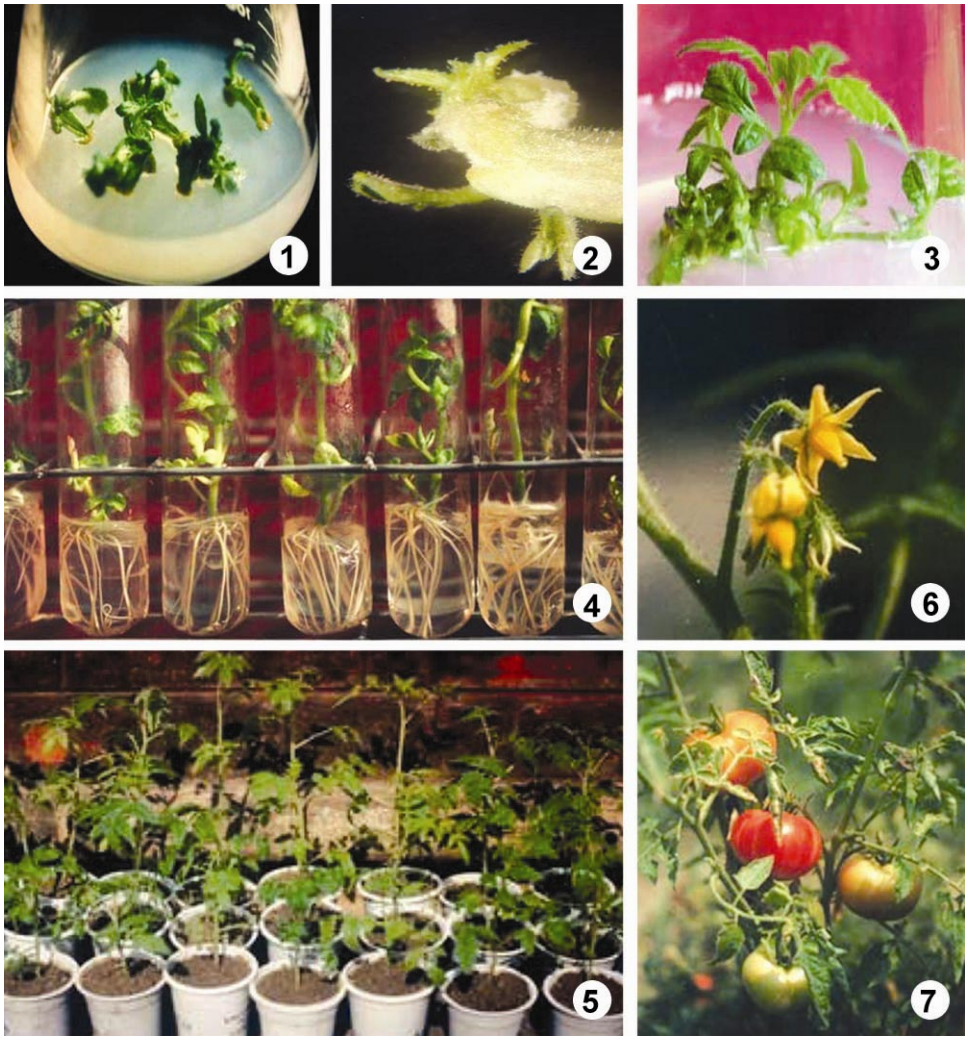
sufficient shoots were obtained in the present study using BAP as a cytokinin supplement for *in vitro* regeneration. Therefore, BAP was chosen as cytokinin supplement for the production of multiple shoots. MS supplemented with different combinations of BAP and Kn were used to examine their synergistic effect on induction of multiple shoots and their development. It was found that number of shoots/explant increase with the increase of BAP concentration but not with the increase of Kn concentration in these combinations. Addition of IAA was found to increase regeneration efficiency as it was earlier reported by Ichimura and Oda (1995), Frary and Earle (1996), Gubis et al. (2003), Cortina and Culianez-Mica (2004).

MS containing hormonal supplements of BAP and Kn with concentrations ranging from 0.5 - 2.0 mg/l and 0.1 mg/l IAA were used for multiple shoot formation in case of both the varieties (Table 1). Induction of shoot was observed within 14 -18 days and maximum shoots/explant were obtained within 60-70 days. Considering the regenerating ability the combination of 1.0 mg/l BAP and 0.1 mg/l IAA was mostly used for shoot regeneration (Figs. 1-3).

As an integral part for successful development of plantlets induction of roots from excised shoots was tried using full or half strength of agar solidified MS medium supplemented with different auxins, *viz.* IAA, IBA and NAA. Half strength of MS medium containig 0.2 mg/l IAA was found to be most effective in producing maximum number of strong and healthy roots (Fig. 4).

Rooted plantlets were transferred to soil where they successfully acclimatized (Fig. 5) and produced flower and subsequently fruits with viable seeds (Figs. 6-7). From the above findings it can be concluded that this regeneration protocol developed in this study is relatively simple, reproducible and genotype independent.

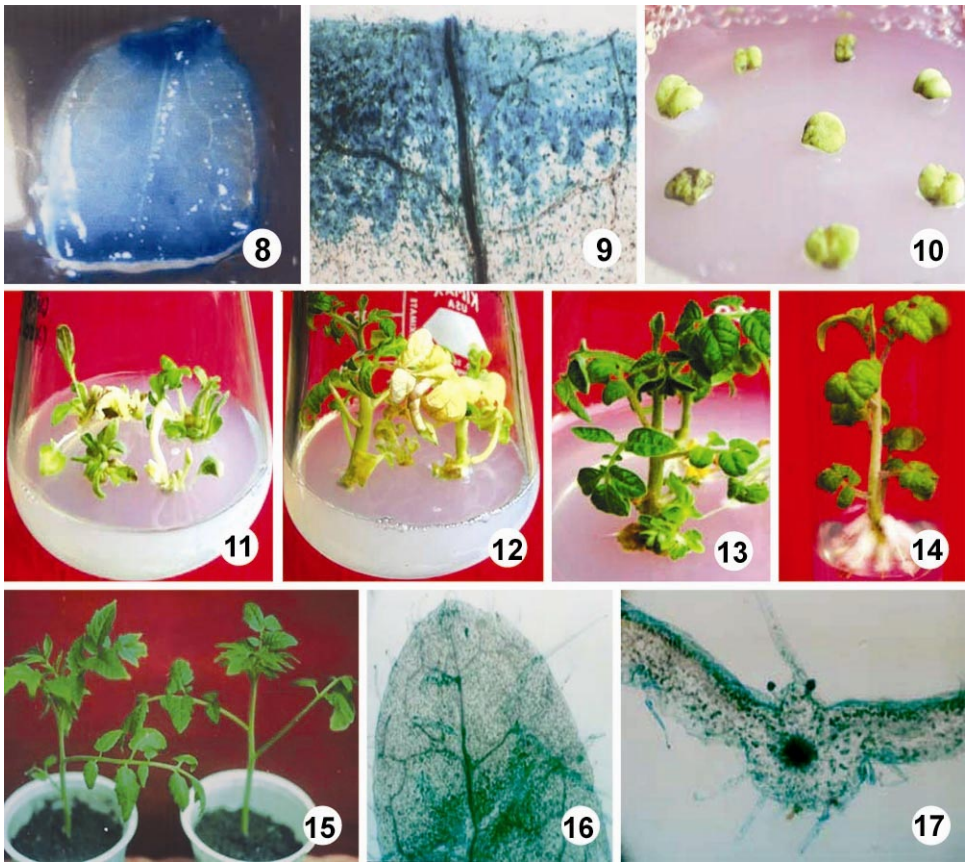
Cotyledonary leaf explants of both the varieties of tomato (PR and BT-3) were found to be compatible with *Agrobacterium tumefaciens* strain LBA4404 (Fig. 8). In most of the cases the entire cut surface of the explants was found to be blue following transient GUS assay (Fig. 9). Several factors influencing *Agrobacterium*-mediated genetic transformation, namely optical density of bacterial suspension, incubation period, and co-cultivation period were optimized during present investigation. In both varieties, increase in incubation and co-cultivation period as well as optical density (OD_{600}) enhanced the percentage of transformed explants. Transformation efficiency of explants, in both the varieties, was found to be maximum when optical density of bacterial suspension was about 0.56 - 0.96 at 600 nm with 30 - 40 min of incubation and three days of co-cultivation period. Similar findings were observed by Ling et al. (1998), Tabaeizadeh et al. (1999), Park et al. (2003) and Cortina and Culianez-Mica (2004).



Figs. 1-7: *In vitro* regeneration in two varieties of tomato, namely, BT-3 and PR. 1. Initiation of multiple shoot formation from cotyledonary leaf explants from PR on MS with 1.0 mg/l BAP + 0.1 mg/l IAA. 2. Stereomicroscopic view of shoot formation in BT-3. 3. Same as Fig. 1 but showing elongated shoots in BT-3. 4. Developing roots from excised shoots of BT-3 on half strength of MS with 0.2 mg/l IAA. 5. Regenerated plantlets of PR in plastic pots containing soil. 6. Flower developed on regenerated plantlet of PR. 7. Same as Fig. 6 but showing developed mature fruits.

The *Agrobacterium* strain used in this investigation (LBA4404/pBI121) has *nptII* gene within its T-DNA and this gene confers kanamycin resistance to the transformed cells. Therefore, selection of the transformants was carried out using various concentrations of kanamycin. It was observed that co-cultivated explants, even in the presence of lower concentrations of kanamycin in regeneration media, failed to regenerate and consequently died (Fig. 10). This observation is

similar to the results obtained in other plant species such as, flax, alfalfa, potato etc. (Pezzotti et al. 1991, McHughen et al. 1989, Cardi et al. 1992). Therefore, selection pressure was not applied immediately after co-cultivation; instead, co-cultivated explants were first allowed to regenerate in regeneration media without any selective agents. After 15 - 20 days the infected cotyledonary leaf explants showing very small shoots and shoot buds were subjected to selection pressure.



Figs. 8-17: 8. Histochemical localization of GUS activity at the cut ends of cotyledonary leaf explants of PR. 9. Same as in Fig.8 but at a magnified view showing the presence of conspicuous blue colour. 10. Infected explants of BT-3 failed to initiate regeneration when the co-cultured explants were directly transferred to the regeneration medium containing 50 mg/l kanamycin. 11. Non-transformed albino shoots on the regeneration medium containing 100 mg/l kanamycin. 12. Surviving shoots of BT-3 (green in colour) in presence of 200 mg/l kanamycin. 13. Survival of healthy shoots following kanamycin selection. 14. Shoot of PR survived through selection pressure producing roots on half the strength of MS with 0.2 mg/l IAA and 100 mg/l kanamycin. 15. Putatively transformed plant (T_0) transferred to plastic pots containing soil. 16. A part of leaf from a transformed plantlet showing stable expression of GUS gene. 17. Transverse section of leaf from transformed plantlets showing expression of GUS gene (× 88).

All control shoots died in the selection medium with 200 mg/l kanamycin (Fig. 11). In this investigation, a lower concentration of kanamycin (50 mg/l) was applied in the initial selection medium and selection pressure was increased gradually in subsequent subcultures. For selection, 50 - 100 mg/l kanamycin was reported to be suitable in obtaining transformed tomato shoots (Ling et al. 1998, Tabaeizadeh et al. 1999, Cortina and Culianez-Mica, 2004). In the present study, the shoots that survived in presence of 150 mg/l kanamycin were subjected to higher selection pressure with 200 mg/l kanamycin to obtain transformants. Shoots, that survived kanamycin selection (Fig. 12), were cultured on MS supplemented with 0.2 mg/l IAA with or without kanamycin to induce roots (Fig. 13). Initiation of proper root formation from the base of the shoots was inhibited in the kanamycin containing media. Mostly adventitious roots were found to initiate from the base of shoots (Fig. 14).

Histochemical GUS assay revealed the presence of blue colored zones in a number of shoots and leaves that survived kanamycin selection. Although a number of randomly selected shoots from initial selection medium showed a positive GUS expression, only a few survived to the final selection pressure. Moreover, all shoots that survived on the final selection pressure did not show positive GUS expression perhaps due to their reversion to non-transgenic state. A number of workers reported such a phenomenon in *Carthamus* (Ying et al. 1992) and in potato (Ottavani et al.1993). These observations indicate that the expression of the GUS activity in the regenerated shoots was not directly correlated with kanamycin resistance. The lack of GUS expression in kanamycin resistant shoots may be due to alteration or loss of GUS gene resulting from rearrangement of the coding sequence or methylation of the gene (Battraw and Hall 1990, Ottaviani et al. 1993).

Rooted plantlets that survived in the final selection phase were transplanted to soil. Histochemical localization of GUS expression was also carried with the fully developed plantlets (T_0) growing in soil (Fig. 15). Conspicuous blue color was observed in leaves of such plantlets (Fig. 16) that indicates the stable integration of GUS gene following application of optimum concentration of kanamycin. Transverse sections from such a leaf also revealed the presence of blue colour (Fig. 17) in the internal tissue.

The transformation experiment indicates that, cotyledonary leaf explants of both the varieties of tomato (PR and BT-3) can be infected by *Agrobacterium tumefaciens* strain LBA4404. The shoot regeneration protocol described here is efficient and reproducible. The selection procedure developed during the study has been found to be effective in recovering transformed plantlets showing that *nptII* gene is an efficient selectable marker for tomatoes (Ling et al. 1998;

Tabaeizadeh et al. 1999; Park et al. 2003; Cortina and Culianez-Mica 2004 and Ieamkhang and Chatchawankanphanich 2005).

In the present investigation, the transgenic nature of the putatively transformed plants was confirmed through GUS histochemical assay only. However, for confirmation of stable integration of transgenes into plants, specific molecular techniques like PCR analysis and Southern hybridization are also necessary. Studies on the inheritance of the transgenic plants are also required to understand the nature of expression of the transgenes. This protocol was primarily developed using screenable marker genes such as GUS and selectable marker genes (*nptII*). Using this protocol, future studies can be conducted to transfer useful candidate genes conferring disease-, insect- and pest resistance in the tomato varieties of Bangladesh.

References

Bartraw MJ and **Hall TC** (1990) Histochemical analysis of CAMV35S promoter β-glucuronidase gene expression in transgenic rice plants. Plant Mol. Biol. **15**: 527-538

Cardi T, Jannamico V, Ambrosio FD, Filippone E and **Lurquin PF** (1992) *Agrobacterium*-mediated genetic transformation of *Solanum commersonii* Dun. Plant Sci. **87**: 179-189.

Cortina C and **Culianez-Mica FA** (2004) Tomato transformation and transgenic plant production. Plant Cell, Tissue and Organ Culture **76:** 269-275.

Costa MGC, Nogueira FTS, Figuiera ML, Otoni WC, Brommonschenkel SH and **Cecon PR (2000)** Influence of antibiotic Timentin on plant regeneration of tomato (*Lycopersicon esculentum* Mill.) cultivers. Plant Cell Rep. **19**: 327-332.

Fariduddin M, Taher A, Islam SMA and **Hossain MZ (2004)** Effect of Variety and Plant Growth Regulators in MS Medium on Shoot Induction from Virus Infected Calli of Tomato. Journal of Biological Sciences **4**(4): 521-526.

Frary A and **Earle DE** (1996) An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. Plant Cell Rep. **16**: 235-240.

Gubis J, Lajchova Z, Farago J and **Jurekova Z (2003)** Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro.* Czech j. Genet. Plant Breed. **39**: 9-14.

Herrea-Esterella L, Depicker A, Van Montagu M and **Schell J** (1983) Expression of chimeric genes transferred into plant cell using a Ti- plasmid derived vector. Nature **303:** 209.

Hooykass PJJB (1988) *Agrobacterium* molecular genetics. Plant Molecular Biology Manual **A 4** : 1-13.

Ichimura K and **Oda M** (1995) Stimulation of shoot regeneration from cotyledon segments of tomato (*Lycopersicon esculentum* Mill.) by agar and its extract. J. Jpn Soc. Hortic. Sci. **64**: 135-141.

Ieamkhang S and **Chatchawankanphanich** (2005) Augmentin as an alternative antibiotic for growth suppression of *Agrobacterium* for tomato (*Lycopersicon esculentum* Mill.) transformation. Plant Cell, Tissue and Organ Cult. **82**: 213-220.

Jefferson RA, Kavanagh TA and **Bevan MW** (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6:** 3901-3907.

Jones JB, Stall RE and **Zitter TA** (1997) Compendium of Tomato Diseases, The APS press. Minnesota. USA. pp. 4-13.

Kartha KK, Gamborg OL, Shyluk JP and **Constable F** (1976**)** Morphogenic investigations on *in vitro* leaf culture of tomato *(Lycopersicon esculentum* Mill. cv. Starfire) and high frequency plant regeneration. ZPflanzen physiol. **77**: 292- 301.

Ling HQ, Krieseleit D and **Ganal MW** (1998) Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobactrerium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). Plant Cell Reports **17**: 843-847.

McHughen A, Jordan M and **Feist G** (1989) A preculture period prior to *Agrobacterium* inoculation increases production of transgenic plants. J. Plant. Physiol. **135**: 245-248.

Oktem HA, Bulbul Y, Oktem E and **Yucel** (1999) Regeneration and *Agrobactrerium*-mediated transformation studies in tomato (*Lycopersicon esculentum* Mill.). Tr. J. of Botany. **23**: 345-348.

Ottaviani MP, Smits T and **Hanish CH** (1993) Differential methylation and expression of the β-glucuronidase and neomycin phosphotransferase genes in transgenic plants of potato cv. Bintje. Plant Science. **88**: 73-81.

Park SH, Morris JL, Park JE, Hirschi KD and **Smith RH** (2003) Efficient and genotype-independent *Agrobactrerium*-mediated tomato transformation. Plant Physiol. **160**(10): 1253-1257.

Pezzotti M, Pupilli F, Damiani F and **Arcioni S** (1991) Transormation of *Mediacgo sativa* L. using a Ti- plasmid derived vector. Plant Breed. **106**: 39-46.

Rojas A, Kvarnheden A and **Valkonen JPT** (2003) Geminiviruses infecting tomato crops in Nicaragua. Plant disease **84**(8): 843-846

Sheeja TE, Mondal AB and **Rathore RKS** (2004) Efficient Plantlet Regeneration in omato (*Lycopersicon esculentum* Mill.). Plant Tissue Cult**. 14**(1): 45-53.

Tabaeizadeh Z, Agharbaoui Z, Harrak H and **Poysa V** (1999) Transgenic tomato plants expressing a *Lycopersicon chilense* chitinase gene demonstrate improved resistance to *Verticillum dahliae* race 2. Plant Cell Reports **19**: 197-202.

Tiwari RN and **Choudhury B** (1986) Solanaceous crops-Vegetable Crops in India. (Ed. by Bose, T. K. and Som, M.G.). pp. 248-292.

Varma A and **Malathi VG** (2003) Emerging geminiviral problems: A serious threat to crop production. *Ann. appl. Biol.* **142:** 145-164.

Ying M, Dyer WEB and **Gergman IW** (1992) *Agrobacterium tumefaciens*-mediated transformation of safflower (*Cathamus tinctroius* L.). Plant Cell Reports **11**(11): 581-585.