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ESTABLISHMENT OF OPTIMAL CONDITIONS FOR AN *AGROBACTERIUM*-MEDIATED TRANSFORMATION IN FOUR TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) VARIETIES GROWN IN BANGLADESH

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ABSTRACT

An efficient transformation protocol for tomato by reducing complexity and increasing transformation efficiency in four varieties namely, Bina tomato-3, Bina tomato-5, Bahar and Pusa Ruby. Transformation of cotyledonary leaf explant was performed with *Agrobacterium tumefaciens* strain LBA4404, harboring binary vector pBI121 having GUS and *nptII* marker genes. Frequency of transient GUS expression showed that the transformation competence in tomato was highly influenced by several factors, like optical density of *Agrobacterium* suspension, incubation period, co-cultivation period etc. In the present study, cotyledonary leaf explant from all four tested varieties found to be efficiently transformed by bacterial suspension having optical density (OD_{600}) of 0.79 with 15 min incubation and 3 days of co-cultivation period. All these conditions along with pre-culture of explants prior to transformation gave better regeneration response following *Agrobacterium* infection. Moreover, for successful regeneration of transformed shoot 200 mg/l kanamycin was found to be most effective selection pressure. As all four varieties showed similar response the present protocol can be considered as a simple and genotype-independent reproducible protocol.

Key words: Solanaceae, Transformation parameter, GUS expression, Cotyledonary leaf explant

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an economically important vegetable crop world-wide. It belongs to a commercially important family, Solanaceae. Due to high food value, the demand of tomatoes is increasing day-by-day. But, abiotic stresses, like salinity, heat, drought, and nutrient deficiencies often constrain its fruit productivity and quality (Zhu 2002, Bhatnagar-Mathur *et al.* 2008, Gao *et al.* 2009). Moreover, many pests, like virus, bacteria, fungi and insects cause considerable damage to this nutritious fruit (Sarker *et al.* 2009).

Plant biotechnological techniques have been successfully applied for developing abiotic (Wangxia *et al*. 2003) and biotic stress (Islam 2006) resistance through genetic transformation in various crops. Among the various methods available, *Agrobacterium*-

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mediated genetic transformation technique is comparatively less expensive and very effective. As a result, this technique is widely used. But in case of tomato transformation, a large number of factors, such as, plant genotype (Frary and Earl 1996**,** Park *et al.* 2003), explants type (Chyi and Phillips 1987, Sun *et al.* 2006), use of feeder layer (Qiu *et al.* 2007), *Agrobacterium* strain and its density (Islam 2007), addition of phenolic compounds (Sun *et al.* 2006), infection and co-cultivation duration (Fillatti *et al.* 1987**,** Hamza and Chupeau 1993, Park *et al.* 2003, Cortina and Culiáñez-Macià 2004) etc. have been reported to influence the transformation efficacy.

Tomato, which is considered as a genetic model for improving dicotyledonous plants is still regarded as a recalcitrant crop for transformation (Ling *et al*. 1998, McCormick 1991). The average transformation frequency ranged from 6.2 - 10.4% depending on regeneration protocol, transformation procedure and genotype (Ellul *et al*. 2003). With the same protocol, change in the genotype reduced the efficiency to 1.8% from the above rate of success (Cortina and Culiáñez-Macià 2004). The absence of highly efficient transformation methodology is the major difficulty in obtaining transgenic tomato. Reports on transformation of Bangladeshi tomato varieties are limited. Begum and Mia (1993) reported tomato regenration in Bari varieties, while Sarker *et al.* (2009) reported transformation in Bari tomato-3 and Pusa Ruby where they observed chimarism of the marker *nptII* gene.

Therefore, in the present study, using marker gene, attempts have been made to develop a transformation protocol for locally grown popular winter and summer tomato varieties along with imported variety Pusa Ruby which is commonly grown in Bangladesh. To achieve this various transformation factors like, OD_{600}, incubation period for infection, preculture, co-cultivation periods, kanamycin concentrations for transformed shoot regeneration have been optimized in all these varieties to establish a simple, genotype independent, efficient and reliable transformation methodology.

MATERIALS AND METHODS

Seeds of four varieties of tomato (*Lycopersicon esculentum* Mill.) namely, Bahar (BR), Bina tomato-3 (B-3), Bina tomato-5 (B-5) and Pusa Ruby (PR) were used in this study. Among these B-3 is a summer variety, while B-5 and Bahar BR are winter varieties of Bangladesh. Seeds of Bina varieties were collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymansing while seeds of the popular Indian variety, Pusa Ruby was collected from the local market.

Seeds of all four varieties were surface sterilized for 1 min with 70% ethanol, followed by 5.25% Clorox treatment with two drops of tween 20 and shaken continuously for 5 - 8 minutes. Seeds were then repeatedly washed with distilled water and kept in a rotator shaker (EDISON; NJ, U.S.A) for 24 - 36 hr in 150 rmp at 28°C while immersed in sterile distilled water. Afterwards seeds were placed on MS

(Murashige and Skoog 1962) medium for germination. Subsequent cotyledonary leaves were excised from 8 - 10 days old seedlings and were transversely cut into two to three segments for use as explants.

Genetically engineered *A. tumefaciens* strain LBA4404 containing plasmid pBI121 of 14KDa (binary vector) was used for infection in the transformation experiments. This binary vector contains the *udiA* gene (Jefferson *et al.* 1987) encoding GUS (β-glucuronidase) enzyme and the *nptII* gene (Hoekema *et al.* 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, both driven by the NOS promoter and NOS terminator.

Overnight grown liquid *Agrobacterium* culture was used for infection of cotyledonary leaf explants. Prior to infection optical density of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Analytikjena Specord 50, Germany). Bacterial suspensions with optical densities of 0.79, 0.64 and 0.42 were used. The explants with or without pre-culture were immersed in the *Agrobacterium* suspension for infection for different time periods, ranging from 10 to 20 minutes and co-cultured for two - three days in dark.

For selection of transformed cells to regenerate into shoots various concentrations of kanamycine (50, 100, 150, 200 and 300 mg/l) was evaluated to determine the best selection pressure in regeneration media. Sensitivity of the explant tissue was compared with control having no kanamycin. Each experiment was performed thrice with 15 explants.

Following co-cultivation the explants were subjected to transient GUS histochemical assay to score the efficiency of transformation. From each batch of transformation experiment, randomly selected 50 - 60% treated explants were examined for GUS activity by GUS histochemical assay (Jefferson *et al.* 1987). The explants were submerged in the substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) and incubated at 37ºC for two - three days. They were then washed in 70% alcohol and scored for transient GUS expression. A characteristic blue color as a result of GUS gene expression in the plant tissue was recorded as a positive result. Each experiment was performed in triplicate and proper control was done with explants not infected with Agrobacterium. As a positive control transformed tobacco plant having nptII in the genome was used.

Two-way ANOVA was performed considering either optical density or incubation period with different tomato varieties as two factors. All statistical analyses were performed using the GraphPad Prism version 5.00 (www.graphpad.com). p value was considered at the 5% level of significance to deduce inference of the significance of the data.

RESULTS AND DISCUSSION

Determination of strain-cultivar compatibility: During evaluation of interaction between *A. tumefactions* strain LBA4404 with the cotyledonary leaf explants of Bina tomato-3 (B-

3), Bina tomato-5 (B-5), Bahar (BR) and Pusa Ruby (PR) varieties, all the explants found susceptible towards infection. GUS histochemical assay revealed B-3 variety to be most susceptible followed by B-5 and PR varieties. Histochemical test showed that BR variety showed lowest infection rate among the four varieties tested. GUS positive regions were detected predominantly along the entire cut ends (Figs 1-3). Similar compatible interaction with this *Agrobacterium* strain was reported in many other tomato varieties (McCormick *et al.* 1986, Cortina and Culiáñez-Macià 2004).

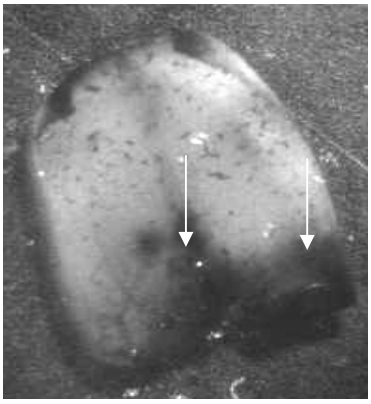

Fig. 1. GUS positive zone in BR predominantly at the cut surface (arrow) of the cotyledonary explants following infection with *Agrobacterium* strain LBA4404. (×8)

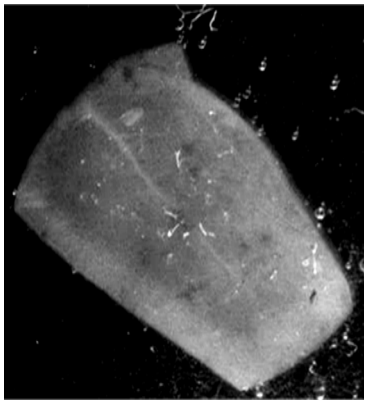

Fig. 2. Negative control of transformation experiment having no GUS positive zone in BR variety. (×8)

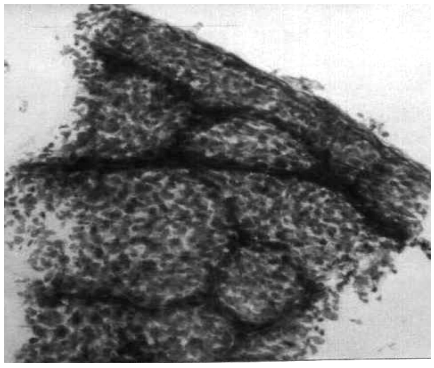

Fig. 3. GUS positive zone at the epidermis and underneath tissue of the co-cultured explants (B-3) following transformation under optimum condition. (×33).

Influence of optical density of Agrobacterium suspension: Bacterial suspensions with optical densities of 0.79, 0.64 and 0.42 were used. Among the various optical density (OD_{600}) tested, the transformation efficiency examined through transient GUS expression revealed significant differences ($F(2, 6) = 16.09$, $P = 5.14$) in the three ODs. It was observed that maximum transformation (~95%) was observed at OD_{600} of 0.79 while minimum (≤ 50%) was at OD_{600} of 0.42 in all varieties **(**Fig. 4**)**. Transformation frequency was found to be increased with the increase of optical density of the

Agrobacterium suspension. Similar trend was reported by Sharma *et al.* (2009) in three Indian varieties, *viz.* Pusa Ruby, Sioux and Arka Vikas, and also by Sarker *et al.* (2009) in Bari tomato 2 and Pusa Ruby. In contrast to these reports, Qiu *et al.* (2007) diluted an overnight culture of *Agrobacterium* strain EHA101 to OD_{600} of 0.2 to get optimum infection condition. This difference may be due to the variation in bacterial strain, because EHA strains are considered supervirulent due to the presence of extra copy of *vir* gene within the cell while LBA strain are moderate virulent.

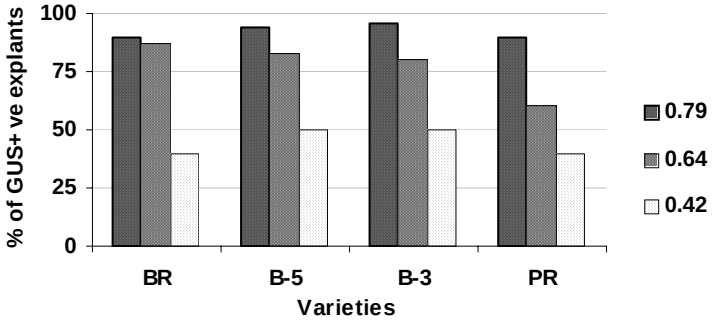

Fig. 4. Percentage of transient GUS expression of transformed explants of BR, B-5, B-3, PR at 0.79, 0.64 and 0.42 of *Agrobacterium* suspension at 600 nm.

Influence of incubation period on transformation: The incubation period was also found to influence transformation competence in all four tomato varieties. Among the incubation period examined, the transformation efficiency showed significant differences ($F(2, 6) = 206.7$, $P = 5.14$) with the three different incubation periods. At OD_{600} 0.79, transient assays showed cent per cent transformation (GUS+ve) in BR and B-3 when explants were incubated for 15 min. In the same condition, B-5 and PR also showed highest (92 and 90%, respectively) GUS positive result. But the GUS positive response was much lower at 20 min of incubation period. Thus, increase in incubation period beyond a critical time length resulted in decrease in transformation efficiency in all the tested varieties (Fig. 5). Though there is no previous report of such tendency, Gao et al. (2009) reported 15 min incubation to be effective for infection. However, Cortina and Culiáñez-Macià (2004) and Sarker et al. (2009) preferred prolonged infection time to achieve the same result.

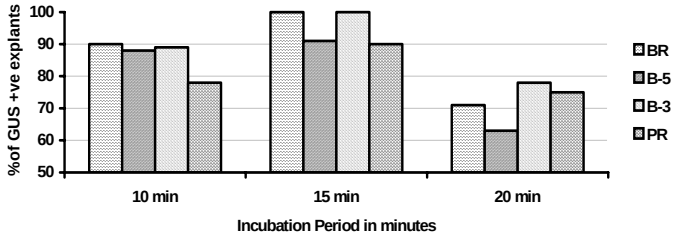

Fig. 5. Percentage of transformed explants in BR, B-5, B-3 and PR following various incubation periods (10, 15 and 20 min).

Influence of pre-culture: In the present study, transformation efficiency was not found to be influenced by pre-culture of explants in any of the four varieties **(**Table 1). This is supported by several reports where different cultivars of tomato were used (Ahsan *et al.* 2007, Islam 2007).

Table 1. Influence of pre-culture on transformation efficiency and subsequent regeneration of cotyledonary leaf explants of BR, B-5, B-3 and PR.

Varieties	% of GUS+ve explants		Days required for regeneration initiation	
	Pre-cultured	Non pre-cultured	Pre-cultured	Non pre-cultured
BR	99	100	15-18	20-25
B-5	90	92	16-19	22-25
B-3	100	100	15-20	23-27
PR	90	90	15-18	20-30

Total of 20 explants used in each case.

Contradicting these reports McCormick (1991) and Gao *et al.* (2009) reported pre-culture to improve tomato transformation frequency. In the present study, pre-cultured explants were found to regenerate faster and performed better than the non-pre-cultured explants (Figs 6-7). Contrary to our finding Hamza and Chupeau (1993) stated pre-culture to stimulate transformation, while reducing the regeneration capacity of the transformed tomato cells. These diverse observations may be due to variation of tomato genotypes.

Fig. 6. Regeneration on non-pre-cultured explants of B-3 following transformation. Photograph taken four weeks after initiation of culture. (×4)

Fig. 7. Regeneration on pre-cultured explants of B-3 following transformation. Photograph taken four weeks after initiation of culture. (×4)

Influence of co-cultivation period: Duration of co-cultivation was found to influence the transformation efficiency and subsequent regeneration capacity. It was found that, percentage of transformation could be increased just by increasing the co-cultivation period while keeping the bacterial density and incubation period constant. But, prolonged

co-cultivation period (more than three days) was found to promote overgrowth of bacteria on the infected explants and also explants found to suffer from poor health showing browning at the cut surfaces. Finally, these explants failed to regenerate. Correspondingly the transformation percentage was found to decrease with the decrease (less than three days) of co-cultivation period (Table 2). Therefore, three days of co-cultivation was determined to be the best for all four varieties tested. Similar duration was adopted by several reports (Park *et al.* 2003, Cortina and Culiáñez-Macià 2004, Islam 2007, Gao *et al.* 2009) though they used longer infection time, different explants and tomato varieties.

Table 2. Influence of co-cultivation periods on transformation of cotyledonary leaf explants of BR, B-5, B-3 and PR in two different bacterial density.

Variety	OD_{600} of bacterial suspension	Co-culture period (hrs)	No. of explants Infected	No. of explants assayed for GUS expression	% of GUS + ve explants
BR	0.79	72	30	10	90
		48	15	5	80
	0.64	72	20	8	88
		48	10	5	40
B-5	0.79	72	40	18	95
		48	15	10	70
	0.64	72	30	12	83
		48	20	6	50
B-3	0.79	72	40	25	96
		48	15	10	70
	0.64	72	35	10	80
		48	30	8	50
PR	0.79	72	20	10	90
		48	15	10	70
	0.64	72	10	5	60
		48	10	5	40

All explants were subjected to 15 min incubation in Agrobacterium suspension.

Kanamycin tolerance test for selection of transformed tissue: Sensitivity of explants towards different concentrations of kanamycin (50, 100, 150, 200 and 300 mg/l) was evaluated. It was found that, in the control set of experiment (without kanamycin), all the incubated explants survived while survival percentage of the explants decreased gradually with the increase of kanamycin concentrations in the regeneration media. The optimum kanamycin concentration was found to be 200 mg/l for the selection of transformed shoots. Similar result was reported by McCormick (1991); Ling *et al.* (1998), Cortina and Culiáñez-Macià *et al.* (2004) and Islam (2007) for positive selection of the transformed tissue. The suggestion of McCormick (McCormick, 1991) not to decrease kanamycin concentration to obtain large number of transformed shoots was found recommendable in the present study.

Present authors' transformation experiments revealed that transformation frequency in tomato is strongly influenced by various factors. The methodology presented here is simple, repeatable and gives high frequency of transformation. Most importantly this protocol is made considering the highest transformation frequency without compromising regeneration capacity to ensure large amount of transgenic plant development. Several reports on tomato regeneration have already been made (Begum and Mia 1993, Chowdhury 2009, Sarker *et al.* 2009). In future, using this transformation coupled with the regeneration protocol already established, attempts will be made to develop transgenic tomato to improve our varieties for better yield and quality.

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