Agrobacterium-mediated genetic transformation in chili cultivar

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Abstract: An investigation was carried out to optimize a protocol for genetic transformation in chili (*Capsicum annuum* L.) through standardization of several experimental factors. These included explants type, inoculation time and co-cultivation time. Cotyledon and hypocotyl callus of Balujuri marich were transformed with *Agrobacterium tumefaciens* LBA4404 strain containing p-glucuronidase (*gus*) gene in binary vector pBI 121 along with kanamycin resistance gene i.e. neomycin phosphotransferase-ll (*np-ll*) for selection in both bacteria and plant. After co-cultivation, only the transformed cells were able to grow on the selective medium. Transformed calli were selected on the selective medium containing 50 mg/l kanamycin. The presence of foreign gene (*gus*) was demonstrated by the expression of chimeric bacterial gene that encode β-glucuronidase. Most of the calli selected on the selective medium from cotyledon and hypocotyl explants were GUS positive.
Key words: Chili, genetic transformation, binary vector, β-glucuronidase gene, *npt-ll*, gene expression.

Introduction

The use of transgenic plants is a method to improve existing chili (*Capsicum* sp.) cultivar. But, application of genetic engineering in chili has been limited because of the difficulties in plant regeneration (Liu *et al.*, 1990). *Capsicum* members have been shown to be recalcitrant to differentiation and plant regeneration under *in vitro* conditions, which in turn makes it very difficult or inefficient to apply recombinant DNA technologies via genetic transformation aimed at genetic improvement against pests and diseases. Although tremendous efforts have been invested world-wide in the direction of transgenic pepper breeding, in most cases only transformation procedures of specific hot pepper cultivars have been achieved (Lee *et al.,* 1993). Recently, the successful transformation of pepper using *Agrobacterium tumefaciens* has been reported (Kumar *et al.*, 2009). This culture is susceptible to many pathogens, however, no success has been obtained because the protocols failed to reproduce the results. The high level of effort invested into transferring genes into chili explants has not yet yielded a successful and reproducible transformation method. Consequently, relatively few studies have been undertaken to explore methodology (Li *et al.,* 2003). Cotyledons or hypocotyls have been the most common source of explants, and the composition of the selection media and culture conditions are similar. Taken together, the procedures described in the literature for chili pepper transformation are not very helpful in terms of achieving routine transformation. To the contrary, successful cases are regarded as rare events. Two major factors clearly inhibit efficient transformation of chili: (a) the shoot regeneration rate of peppers is genotype-dependent (Christopher and Rajam, 1996) and genotype specificity affects the transformation rate; (b) the very low efficiency of transformation. The majority of earlier investigations reported the occasional successful transformation of one or two plants but not continuous success with different genes and lines and the production of many transformed plants (Manoharan *et al.,* 1998). The efficiency of transformation and production of transgenic plants depends on the establishment of optimal protocols for inoculation and subsequent selection and regeneration of transformed cells (Draper *et al*., 1988). Using established regeneration protocol for chili, the objective of this research is to develop procedures to introduce a foreign gene using *Agrobacterium*-mediated genetic transformation by infection of cotyledon and hypocotyls explant in locally available chili varieties.

Materials and Methods

Genetic transformation protocol development was carried out in Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh during the period of 2009-2010. Cotyledon and hypocotyls calli of local chili variety Balujuri marich were used as plant material. Genetically engineered *A. tumefaciens* strain LBA4404 was used for infection in the pre-cultured explants. In this experiment, cotyledon and hypocotyls explants of chili were co-cultivated with *Agrobacterium*, resistant to kanamycin containing CBL Interacting Protein Kinase (*CIPK*) drought resistant gene construct obtained from Dr. Narendra Tuteja, Associated Scientist, Plant Molecular Biology, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. The calli developed and the plantlets regenerated after co-cultivation was sub-cultured in selection medium (kanamycin and cefotaxime supplemented) for identification of transformed plantlets. Three factors, two explant (cotyledon, hypocotyls callus), three inoculation time (10, 15 and 20 minutes) and three co-cultivation time (24, 48 and 72 hours) were applied in the experiment. Total 18 treatments were set in CRD design and each treatment consisted of four vials and replicated 3 times. Transformation and plant regeneration: Chili seeds were sterilized in a 2% sodium hypocloride solution for 20 minutes, washed three times in distilled water and then grown on MS medium in a culture room maintained at 25°C, a light intensity of 1800 lux ana a16/8-h (day/night) photoperiod. A single colony of *A. tumefaciens* strain LBA 4404 was inoculated in 20 ml LB liquid medium supplemented with 50mg/l km, 100mg/l 100 mg/L cefotaxime and cultured on a rotary shaker at 28°C, 180 rpm. For 48 hour. The bacteria cell were than suspended in MS liquid medium to O.D.600=0.38-0.42. Cotyledon explants were cut from 12 to one day old seedlings, following pre-culture on MS medium supplemented with 5.0mg/L BAP and 0.3mg/L NAA were used for induction and maintenance of callus. The explants were inoculated with the cultures of Agrobacterium strain LB4404 10-20 minutes followed by co culture on 5.0mg/L BAP and 0.3mg/L NAA. MS media

supplemented with 5 mg/L BAP, 0.3 mg/L NAA, 20 mg/L kanamycin and 100 mg/L cefotaxime and MS media supplemented with 5 mg/L BAP, 0.3 mg/L NAA, 30 mg/L kanamycin and 100 mg/L cefotaxime were used as low and high selection medium respectively for post-cultivation and regeneration. The GUS (β-Glucuronidase) straining assay solution was prepared with X-gluc (solvent: DMSO) 8.89mg/10ml, Chloramphenicol 200 µl./10ml, NaH_2PO_4 119.8mg/10ml, Triton × (10%) 100 µl,/10ml Methanol 2ml/10ml. P^H was adjusted at 7.0- 8.0 by adding P^H-10 buffer solution. From each batch of calli following each transformation experiment, randomly selected survived calli were examined for GUS histochemical assay. For this test survived calli were immersed in X-gluc (5-bromo-4-chloro-3-indoly-1-glucuronide) solution and were incubated at 37^0C for overnight. A characteristic blue color would be the expression of GUS (*β-Glucuronidase*) gene in the plant tissue. Proper control for GUS histochemical assay was done with the explants having no *Agrobacterium* infection. After X-gluc treated explants were transferred to 70% alcohol for degreening. Following degreening, explants were observed under stereomicroscope (Begam, 2007). Data of days number of survived callus, % survived callus (number of survived callus/total number of cultured callus × 100), number of callus positive for GUS assay and percentage of callus positive for GUS (Percent GUS expression) assay was counted. The data was analyzed following standard statistical procedures (Gomez and Gomez, 1984) and mean differences were adjusted by Duncan's Multiple Range Test (DMRT) using a computer operated programme named MSTST-C.

Results and Discussion

Genetic transformation is a powerful and important tool for crop improvement, which offers the possibility to introduce the desired trait into the elite genotype without changing any other attributes to the transformed genotype. Chili is known as a crop which is very difficult to be transformed (Christopher and Rajam,1996).Because several factors influence *Agrobacterium*-mediated genetic transformation. In the present study transformation of cv. Balujuri marich was studied Inoculation time, Co-cultivation time, Kanamycin resistance was recorded during the experiment.

Table 1. Effect of explant, inoculation time and co-cultivation time on number and percentage of survived callus and GUS histochemical assay

Treatments	Number of survived callus	Percent of survived callus	Number of GUS + ve callus	% of GUS + ve callus
Explant				
Cotyledon	10.94	43.78	2.75	45.83
Hypocotyl	9.89	39.56	2.25	37.50
Inoculation time				
10 mins.	9.92	39.67	2.38	39.58
15 mins.	10.79	43.17	2.71	45.14
20 mins.	10.54	42.17	2.42	40.28
Co-cultivation time				
24 hrs	9.58 b	38.33b	1.92 b	31.94 b
48 hrs	11.50 a	46.00a	3.00 a	50.00 a
72 hrs	10.17 b	4067b	2.58 a	43.05 a
CV (%)	7.72	7.72	20.45	20.45

Figures followed by same letter(s) are statistically similar as per DMRT

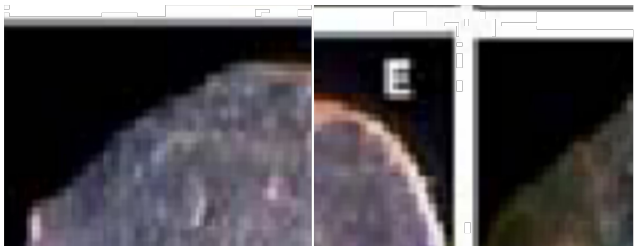

Plate 1. GUS positive tissues (right) and Control (left) tissues of Cotyledon explant

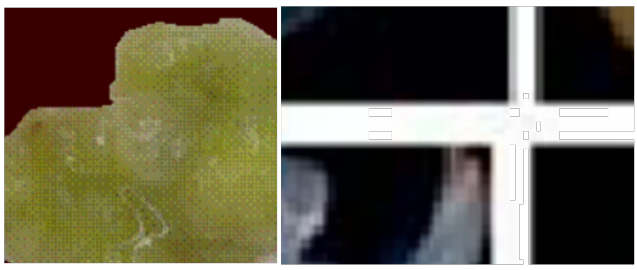

Plate 2. GUS positive tissues (right) and Control (left) tissues of Hypocotyl explant

Effect of explant (callus) on transformation: Effect of explants was observed as significant in all the parameters studied in the experiment. Among the explants cotyledon callus showed the best performance over hypocotyl callus. The highest number (10.94) cotyledon callus survived in co-cultivation medium and the lowest number (9.89) was found in hypocotyl callus. Regarding percentage of survived callus, cotyledon explants showed the best performance (43.78 %) and the lowest survival percentage was found in hypocotyl explants (39.56 %) (Table1 and Plate 2). In an investigation of *Agrobacterium*- mediated genetic transformation of potato, Begum (2005) found that both number and percentage of survived calli per pertridish were higher (4.55 and 25.01%, respectively) in leaf calli than internodal calli (2.33 and 11.16 %, respectively). The highest GUS positive callus was obtained from cotyledon explants (2.75) and the hypocotyl explants gave the lowest number (2.25) GUS positive transformants.

In case of percentage of GUS positive callus, cotyledon explants gave the highest percentage (45.83 %) (Table1 and Plate 1). Nishibayashi *et al.* (1996) reported that very young leaves of more than 50 percent plantlets when treated with x-gluc displayed strong GUS expression. From above findings it was revealed that transformation ability may differ from explant to explant.

Effect of inoculation time on transformation: Inoculation time is an important factor in transformation experiment mediated by *A. tumefaciens*. It was found that there was significant effects of inoculation times viz. 10, 15, and 20 minutes on number of survived callus, % of survived callus but not significant on number of GUS + ve and % of GUS + ve. The best results of the studied parameter were observed from inoculation after 15 min than that of other two inoculation time (Table 1).

Effect of co-cultivation on transformation: Co-cultivation time is an important factor in *Agrobacterium*-mediated plant genetic transformation. Three co-cultivation times selected in the study had showed highly significant effects in all the parameters studied (Table 1). The longer co-cultivation time up to certain limit (48 hrs) showed better performance than shorter or longer one. In the experiment the higher number and percentage of callus (11.50 and 46.00%) respectively, survived when they were kept in co-cultivation medium for two days (48 hrs). The results were in support by Li *et al*., (2003) who showed that a co-cultivation time of two days was optimal for the transformation of pepper cotyledons and hypocotyls, but a longer (3, 4, 5, 7 days) co-cultivation time resulted in the necrosis of explants, which impeded shoot regeneration. In addition, in *Citrus paradisi* the highest number of GUS^+ shoots and segments with GUS^+ shoots were obtained with a two-day co-cultivation time (Costa *et al.,* 2002).

Combined effect of explant, inoculation time and co-cultivation time: The combined effect of explant (cotyledon, hypocotyl), inoculation time (10, 15 and 20 min) and co-cultivation time (24, 48 and 72 hrs) was studied for selecting the best treatment in terms of number of survived callus, % of survived callus, number of GUS +ve callus and % of GUS +ve callus. It was observed that cotyledon explant, inoculated up to 15 min. and co-cultivated for 48 hrs remarked as best treatment as it ensured the best performances in respect of above four studied parameters (Table 2).

Table 2. Combined effect of explant, inoculation time and co-cultivation time on number and percentage of survived callus and GUS histochemical assay

Explant	Infection time	Co-cultivation time	Number of survived callus	Survived callus (%)	Number of GUS +ve callus	GUS +ve callus (%)
Cotyledon explants	10 min	24 hrs	9.50	38.00	1.75	29.17
		48 hrs	11.25	45.00	3.25	54.17
		72 hrs	10.00	40.00	2.50	41.67
	15 min	24 hrs	10.25	41.00	2.25	37.50
		48 hrs	12.50	50.00	3.50	58.34
		72 hrs	11.50	46.00	3.25	54.17
	20 min	24 hrs	10.50	42.00	2.25	37.50
		48 hrs	12.00	48.00	3.25	54.17
		72 hrs	11.00	44.00	2.75	45.83
Hypocotyl explants	10 min	24 hrs	9.00	36.00	1.75	29.17
		48 hrs	10.75	43.00	2.75	45.83
		72 hrs	9.00	36.00	2.25	37.50
	15 min	24 hrs	9.25	37.00	2.00	33.33
		48 hrs	11.25	45.00	2.75	45.83
		72 hrs	10.00	40.00	2.50	41.67
	20 min	24 hrs	9.00	36.00	1.50	25.00
		48 hrs	11.25	45.00	2.50	41.67
		72 hrs	9.50	38.00	2.25	37.50
CV (%)			7.72	7.72	20.45	20.45

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