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A simple and efficient *Agrobacterium*-mediated transformation of tomato

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Abstract : A reproducible transformation system for the tomato genotype IPA-3 using *Agrobacterium tumefaciens* and cotyledon explants was developed. Disarmed *Agrobacterium tumefaciens* - GV 3101 carrying binary vector p35S-2-SFR was used in the genetic transformation study. The binary vector contained GUS under the control of cauliflower mosaic 35S (CaMV35S) promoter. Factors governing the efficiency of *Agrobacterium*-mediated transformation include age of explants, bacterial concentration, pre-culture period and co-cultivation time were optimized. The frequency of GUS expression varied from 2.66 to 23.16 % under different treatments. The *Agrobacterium* (OD_{600}=1) inoculum when diluted to 1:20 recorded maximum GUS expression on treatment duration of 30 minutes. The 10 day old seedling explants i.e. cotyledons also proved superior to transformation over 7 and 12 day old. The effect of 1 day pre-culture was also significant over 2 and 3-day. Similarly, co-cultivation period also showed significant effect on percent GUS expression and thereby transformation efficiency. The present protocol can be considered as a simple and reproducible protocol for obtaining high frequency of transformation in Tomato.

Keywords: Transformation, Agrobacterium,Tomato

Introduction

Tomato (*Solanum lycopersicum* L., 2n=2x=24) is a major vegetable crop of all regions of the world. In India, productivity of tomato is low primarily owing to its vulnerability to various biotic and abiotic stresses. Transgenic approach where crops are so designed to produce their own resistance system seems to be a viable and appropriate approach. Among the biotechnological approaches to develop transgenic plants, *Agrobacterium*-mediated transformation is the most extensively used method. It exploits the natural ability of *Agrobacterium* to transform plants to complete its own life cycle (Otoni *et al.,* 2003). Tomato transformation using *Agrobacterium tumefaciens*, has developed very rapidly since 1985. Factors such as variety (Ultzen *et al*., 1995; Ling *et al*., 1998; Ellul *et al*., 2003), explants material (Pftizer 1998), bacterial concentration (Sharma *et al.,* 2009) and *Agrobacterium* virulent gene inducers (Stachel *et al*., 1986) have been reported to influence transformation efficiency. Therefore, a series of experiments were conducted on the cotyledons of tomato genotype IPA-3 after developing a high frequency plant regeneration system (Devi *et al.,* 2005) to obtain maximum efficiency of transformation using *Agrobacterium* i.e. bacterial inoculum density, explant age (explants), inoculation period, pre-incubation period, and co-cultivation duration.

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Materials and methods

Media preparation and raising of plant material

Seeds of genotype IPA-3 were surface sterilized with commonly used commercial bleach, 'Ala Bleach' for 20 minutes followed by washing with sterile distilled water. The sterilized seeds were cultured on Murashige and Skoog (MS; 1962) basal medium containing thiamine HCl (0.1 mg l^{-1}), pyridoxine HCl (0.5 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), myo-inositol (100 mg l^{-1}), sucrose (3.0 gl^{-1}) and agar (0.8 %). The pH of medium was adjusted to 5.8 by adding 1N NaOH/ 1N HCl solution drop by drop. The *in vitro* germinated seedlings served as an explant source for gene manipulation studies. The explants were cultured on MS medium supplemented with BAP (2.0 mg l^{-1}) and kinetin (1.0 mg l^{-1}). The medium was first

dispensed into glass jars (100 ml per jar). These glass jars were properly capped before autoclaving at 1.05 kg cm^{-1} square pressure and 121°C temperature for 22 minutes. To prevent microbes, all decontamination procedures and aseptic manipulations were performed in a laminar flow hood.

Agrobacterium strain and growth conditions

Disarmed *Agrobacterium tumefaciens* strain GV 3101 carrying binary vector p35S-2-SFR was used in the genetic transformation study. The binary vector contained GUS under the control of cauliflower mosaic 35S (CaMV35S) promoter. *Agrobacterium* strain GV 3101 was maintained at 28°C on solid YEB medium supplemented with appropriate concentrations of selective antibiotics viz rifampicin, gentamycin and spectinomycin. The *Agrobacterium* strain was grown in liquid YEB until an OD_{600}=1.0 was obtained at 28°C in a rotary shaker (250 rpm) for 24 h supplemented with appropriate concentrations of bacterial selective antibiotics. The transformation parameters standardized in the study are presented in Table 1.

Table 1. Different parameters studied for *Agrobacterium* mediated genetic transformation

Bacterial inoculum density (OD_{600} 1.0)	Undiluted (1:1)	1:10	1::20
Seedling age (days)	7	10	12
Duration of treatment (min)	45	30	20
Pre-incubation period (days)	1	2	3
Co-cultivation duration (days)	2	3	4

GUS assay

The *Agrobacterium* treated explants were analyzed histochemically for transient GUS activity, using X-gluc (5-bromo-4-chloro-3-indolyl-?-D-glucuronide). The *Agrobacterium* treated explants i.e. cotyledons were put in to 1 ml filter sterilized GUS substrate. Explants were incubated in GUS substrate for 24 h at 35ºC. The GUS substrate was then decanted off and replaced with 1 ml of 70 % (*v/v*) ethanol to stop the reaction, maintain aseptic conditions and partially clear the tissues. Cells expressing GUS activity were observed as blue under a inverted microscope. Each blue explant was scored as one transformation event.

Statistical analyses

For each treatment total number of GUS positive explants per petri plate were counted. Percent GUS expression was calculated as the number of transformation events over total number of *Agrobacterium* treated explants per petri plate. Statistical analysis was done according to the CPCS-I package using CRD design. CD values at 5% level of significance were calculated and the interpretations were made accordingly.

Results and discussion

Agrobacterium mediated genetic transformation is being preferred for its simplicity, cost effectiveness and ability to deliver usually the single copies of the gene of interest. In the present investigation the following parameters were studied to obtain high frequency of transformation in tomato.

Effect of bacterial inoculum density on percent transformation

Exposure of cotyledonary explants of genotype IPA-3 to an undiluted culture of *Agrobacterium* (OD_{600} =1.0) for 30 minutes and 2 days co-cultivation period resulted in severe necrosis of explants and registered 100% mortality of the explants (Table 2). Growth of *Agrobacterium* completely seized the growth of cotyledons and led to death of explants. Diluted culture at OD_{600} =1.0 (1:10 and 1:20 dilution) reduced necrosis to a great extent and recorded 49.55 and 17.23% mortality, respectively, and differed significantly among each other. The percent GUS response varied significantly among the treatments. The maximum GUS response i.e. 12.03 % was recorded in the 1:20 dilution when treated for 30 minutes which was significantly higher than 1:10 dilution. Islam *et al.* (2010) reported that 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.

Table 2. Effect of *Agrobacterium* inoculum density on percent mortality and GUS expression in cotyledonary explants of tomato treated for 30 minutes

Bacterial inoculum density (OD_{600} = 1.0)	Mortality (%)	GUS expression (%)
Undiluted (1:1) culture	100.00	Nil
1:10 dilution	49.55	10.89
1:20 dilution	17.23	12.03
CD at 5%	4.74	0.43

Effect of Agrobacterium *inoculation durations on percent transformation*

The explant mortality was recorded to be 18.53, 40.88 and 80.93 percent when treated for 30, 45 and 60 minutes. The differences among treatments were significantly different. Percent GUS expression was maximum i.e. 17.53 percent when the explants were treated for 30 minutes duration (Table 3) which was significantly higher over both other treatments. With either increase or decrease in the duration of treatment there was a significant decrease in the percent GUS expression. Similar results were reported by Islam *et al.* (2010) that increase in incubation period beyond a critical time length resulted in decrease in transformation efficiency in all the tested varieties. In contrast, Rai *et al*. (2012) found highest transformation with 5 min, and increase in the transformation frequency with decreasing time. However, they had used different tomato genotypes and transformation conditions. 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.

Table 3. Effect of *Agrobacterium* inoculation duration on the percent mortality and GUS expression in cotyledonary explants of tomato

Duration in minutes	Mortality (%)	GUS expression (%)
45	80.93	2.66
30	40.88	17.53
20	18.53	12.00
CD at 5%	4.40	2.71

Effect of seedling age on percent transformation

Different workers used different age of the seedling explants i.e. 10 day old (Sharma *et al.,* 2009), 8-10 day old (Islam *et al.,* 2010), 15 –day old (Paramesh *et al.,* 2010), and 6 days (Rai *et al*., 2012). Therefore to standardize the effect of age of the explants on percent plant transformation this experiment was undertaken. The data presented in Table 4 revealed that necrotic reaction from 7-day old seedlings was so high that none could survive the treatment. On the other hand, with 10 and 12 day old seedlings, the percent mortality was 25.09 and 14.03 percent, respectively. The maximum GUS response was recorded in 10 day old seedlings which was significantly higher over both of the other age groups. Therefore further experiments were conducted with explants excised from 10 day old seedlings.

Table 4. Effect of seedling age on the explant mortality and per cent GUS expression in *Agrobacterium* treated cotyledonary explants of tomato

Seedling age (days)	Mortality (%)	GUS expression (%)
7	100.00	Nil
10	19.07	18.21
12	14.03	17.04
CD at 5%	3.57	0.50

Table 5. Effect of pre-culture period on explant mortality and GUS expression in the *Agrobacterium* treated cotyledonary explants of tomato

Per-culture period (days)	Mmortality (%)	GUS expression (%)
0	19.33	12.93
1	16.18	13.95
2	15.00	10.77
3	11.30	9.98
CD at 5%	2.22	0.89

Effect of pre-culture period on transformation

Cotyledonary explants of genotype IPA-3 were hyper sensitive to the bacterial culture when no pre-culture was done. A short pre-culture period of one day on the regeneration medium reduced the percent explant mortality significantly and enhanced percent GUS expression by 7.89 percent over the control (Table 5). Further increase in the duration of pre-culture period reduced the percent mortality of explants. However, GUS expression was significantly reduced by 16.7 and 22.82 percent over no pre culture period. But Islam *et al.* (2010) reported that transformation efficiency was not found to be influenced by pre-culture of explant. This can be supported by several reports where different cultivars of tomato were used (Ahsan *et al.,* 2007; Islam 2007). Contradicting these reports McCormick (1991), Gao *et al.* (2009), and Rai *et al*., (2012) 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. 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.

Effect of co-cultivation duration on transformation

Duration of co-cultivation was found to influence the transformation efficiency and subsequent regeneration capacity. Complete mortality was recorded when duration of co-cultivation was increased to four days (Table 6). Two day co-cultivation duration was better over one, three and four day co-cultivation. When co-

Table 6. Effect of co-cultivation duration on explant mortality and GUS expression in *Agrobacterium* treated cotyledonary explants of tomato

Co-cultivation duration (days)	Mortality (%)	GUS expression (%)
1	7.55	11.68
2	17.21	23.16
3	24.93	22.99
4	98.51	Nil
CD at 5%	3.31	2.71

cultivated for three days, elimination of *Agrobacterium* from the explants was difficult whereas in four days co-cultivation there was complete necrosis and death of explants. In one day co-cultivation period, percent mortality was less but the percent GUS expression was reduced considerably. 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 two days) of co-cultivation period. Therefore, two-three days of co-cultivation was determined to be the best for genotype IPA-3. 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. All explants were subjected to 15 min incubation in *Agrobacterium* suspension. Co-cultivation duration has been reported to play an important role in determining the genetic transformation efficiency in cauliflower (Chakraborty *et al.,* 2002) and broccoli (Viswakarma *et al.,* 2004). Transformation experiments of this study 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 (Devi *et al* 2005, Chowdhury 2009; Sarker *et al.,* 2009). In future, using this transformation method coupled with the regeneration protocol already established, attempts will be made to develop transgenic tomato to improve our varieties for better yield and quality.

सारांश

एग्रोबैक्टिरीयम ट्यूमेफेसिएन्स और टमाटर की आईपीए–3 किस्म के बीजपत्रों का प्रयोग करके पुनः उत्पन्न कर सकने योग्य परिवर्तन प्रणाली विकसित की गई। इसके लिए निरस्त्रित एग्रौबैक्टिरियम ट्यूमेफेसिएन्स के जीवी–3101 प्रकार जो कि द्विआधारी वाहक पी35एस–2–एसएफआर का वहन करता था, का प्रयोग आनुवंशिक परिवर्तन के अध्ययन के लिए किया गया। एग्रौबैक्टिरियम मध्यस्थ परिवर्तन की क्षमता को प्रभावित करने वाले कारक जिसमें– बीजपत्र की आयु, जीवाणु की सान्द्रता, पूर्व–संवर्धन की अवधि और सह–संवर्धन की अवधि सम्मिलित हैं, को आदर्शिकृत किया गया। जीयूएस अभिव्यक्ति की क्षमता विभिन्न उपचारों में 2.66–23.16% रही, एग्रौबैक्टिरियम (OD_{600}=1) को जब 1:20 के अनुपात में तनु करके 30 मीन का उपचार दिया गया तो अधिकतम जीयूएस अभिव्यक्ति अभिलिखित की गई। 10 दिन आयु के अंकुर के बीजपत्र 7 और 12 दिन अंकूर के आयु के तुलना में परिवर्तन के लिए श्रेष्ठ सिद्ध हुए। 1 दिन के पूर्व संवर्धन का प्रभाव भी 2–3 दिन के तुलना में महत्वपूर्ण था। इसी प्रकार सह–संवर्धन अवधि भी जीयूएस अभिव्यक्ति और उसके द्वारा परिवर्तन क्षमता पर महत्वपूर्ण प्रभाव प्रदर्शित की। प्रस्तुत पद्धति को टमाटर के परिवर्तन की उच्च आवृत्ति प्राप्त करने के लिये एक सहज और पुनः उत्पन्न करने योग्य पद्धति के रूप में विचार किया जा सकता है।

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