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Factors Influencing Regeneration and Genetic Transformation of Three Elite Cultivars of Tomato (*Lycopersicon esculentum* L.)

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Abstract: In this study the effect of three genotypes (KalG, Kal-early and Su2270 as the commercial cultivars in Iran), two explants (cotyledon and hypocotyl), growth regulators (BAP, NAA and Zeatin) and a strain of *Agrobacterium tumefaciense* (pGV3850) on regeneration and transformation efficiency is reported. Although all cultivars and explants were regenerated on the different media, however our results showed that there were significant differences between genotypes and explants in the number of shoot formation per explant. Optimal regeneration of KalG, Kal-early and Su2270 cultivars were observed on MS media supplemented with 2 mg L^{-1} Zeatin and 2 mg L^{-1} BAP, respectively. The maximum number of shoot formation per explant was observed in cotyledon and hypocotyle explants in KalG and Su2270 cultivars, respectively. The transformation rate was 17% for Kal-early to 35% for KalG cultivars. Transformation were confirmed by Gus assay and PCR analysis.

Key words: Agrobacterium, tomato (*Lycopersicon esculentum*), transformation

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the most important vegetable crops in the world as a source of vitamins, karoteins, amino acids, sugars and other substances that have an important role in human health. Since the first report of tomato transformation (McCormick *et al*., 1986), there have been numerous publications of genetic manipulation on several cultivars of tomato (Chi and Philips, 1987; Fillatti *et al*., 1987; Fischhoff *et al*., 1987; Delnnay *et al*., 1989; Van Roekel *et al*., 1993; Agharbaoui *et al*., 1995; Frary and Earle, 1996; Ling *et al*., 1998; Tabaeizadeh *et al*., 1999; Vidya *et al*., 2000; Hu and Phillips, 2001). However, the transformation of the tomato using *Agrobacterium tumefaciens* is highly dependent on genotypes. Lack of efficient regeneration procedures is a major obstacle in the application of gene transfer technology to economically important tomato cultivars (Velcheva *et al*., 2005). Until now different explants such as leaf disks (McCormick *et al*., 1986), cotyledon (Van Roekel *et al*., 1993), protoplasts (Roset and Gilissen, 1989) and hypocotyls (Park *et al*., 2003) were used for tomato transformation. Different concentrations of BAP, IAA, NAA and Zeatin were used for direct shoot regeneration in tomato cultivars (Park *et al*., 2003; Velcheva *et al*., 2005). Also, different factors were found to play major roles in tomato transformation: *Agrobacterium* cell density (Velcheva *et al*., 2005), regeneration and co-cultivation conditions (Drorri and Altman, 2001), acetosyringone and cell competence after wounding and bacterial strain (Drorri and Altman, 2001). All these studies indicated that the regeneration efficiency and transformation of the tomato is highly dependent on the genotype and needs to be established for each cultivar (Velcheva *et al*., 2005).

Therefore, the aim of this study is to identify the various factors involved in the regeneration and transformation of three high yielding (70-80 ton h^{-1}) commercial varieties of the tomato (KalG, Kal-early and Su2270) that also have some other good qualities like resistance to many disease (TMV, CMV virus and some fungal or insect pathogen) and tolerance against for various biotic and abiotic stresses which make them important and widely grown cultivars in Iran.

MATERIALS AND METHODS

Plant materials: This study was done in the Plant Molecular Department of NRCGEB during the years 2004-2005. In this study, seeds of *Lycopersicon esculentum* Mill. KalG, Kal-early and Su2270 cultivars

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were kindly provided by seed bank of Horticulture Department of Ferdowsi University in Mashad. Seeds of the tomato were first surface-sterilized in 70% (v/v) ethanol for 30 sec followed by 1% sodium hypochlorite solution for 10 min and rinsed 4 times with sterilized water. Seeds were then germinated on a MS inorganic salt medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose, pH 5.7 and solidified using 8 g L^{-1} TC agar (Sigma, St. Louis, MO). All cultures were maintained at 25°C under fluorescent light at a 16 h light/8 h dark cycle. Shoot tips of cotyledon and hypocotyls removed from 11 day-old seedlings were used as explants for regeneration. Cotyledons were then pierced with needles in order to increase their infection rate. Cotyledon segments were placed upside down in petri dishes containing preculture medium and incubated for 2 days at 25°C in dark.

Culture media for regeneration: MS medium containing MS inorganic salts (Murashige and Skoog, 1962), 3% (w/v) sucrose, 100 mg L^{-1} inositol and MS vitamins with the following plant growth regulator concentrations were tested for callus induction and shoot initiation (Table 1).

Bacterial strain and plasmid: *Agrobacterium tumefaciens* strain, *GV3850,* harboring the binary vector pBI121 was used for this study. The bacteria were grown overnight in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} trypton and 10 g L^{-1} NaCl) with 50 mg L^{-1} kanamycin at 28°C on a rotary shaker (220 rpm) and then diluted to OD_{600} = 1.0. Bacterial suspensions were centrifuged at 3000×g for 15 min in a 50 mL falcon tube, resuspended in liquid MS medium and finally used for co-cultivation.

Transformation procedure: Tomato explants were removed from the preculture medium and placed into sterile glass jars, containing the bacterial suspension and gently shaken for 5 min. Explants were blotted dry on sterile filter paper and cultivated on a co-culture medium at 25°C. After 2 days, explants were immersed in a washing medium (MS salts + 30 g L^{-1} sucrose + 500 mg L^{-1} cefotaxime), blotted dry on sterile filter paper and transferred to shoot Regeneration Media (RM) (Table 1) with 50 mg L^{-1} kanamycin at 25°C under fluorescent light at a 16 h light/8 h dark cycle. Explants were subcultured every 2 weeks. After four weeks of culture, the percentage of shoot formation per explants was evaluated in two different media and three genotypes. After 5-6 weeks, elongated shoots, emerged from the cut surfaces, were excised and placed on shoot Elongation Medium (EM) (Table 2). Most of the elongated shoots regenerated into plants and formed roots on MS basal medium lacking growth regulators (RTM).

Table 1: Different culture media with various plant growth regulators used for tomato regeneration

Media culture (mg L^{-1})						
PGR	M1	M2	M3	M4	M5	M6
BAP	1	2	2	2	-	-
NAA	0.1	0.1	0.5	-	0.1	-
ZR	-	-	-	-	2	2

Table 2: Media compositions utilized for *Agrobacterium*-mediated transformation and regeneration of the tomato

Media	GM[b]	PM[c]	CM[d]	RM^{e}_{1}	RM^{e}_{2}	EM[f]	RTM[g]
MS[a]	+	+	+	+	+	+	+
Sucrose (g L^{-1})	30	30	30	30	30	30	30
MS vitamins	+	+	+	+	+	+	+
Agargel(g L^{-1})	8	8	8	8	8	8	8
BAP (mg^{-1})		1	1	2			
Zeatin (mg^{-1})					2		
NAA (mg^{-1})		1	1				
Cefotaxime (mg^{-1})				150	150	150	100
Kanamycin (mg^{-1})				50	50	100	100
pH	5.7	5.7	5.7	5.7	5.7	5.7	5.7

a: Murashige and Skoog, b: Germination medium, c: Preculture medium, d: Co-culture medium, e: Regeneration medium, f: Elongation medium, g: Rooting medium

Molecular and histochemical analysis: GUS enzyme activity of transgenic shoots were determined 2 to 8 weeks after transformation by histochemical assay as described by Jefferson (1987). The total genomic DNA was extracted from leaves using the CTAB method (Saghai-Maroof *et al*., 1984). Polymerase Chain Reaction (PCR) was used to detect the presence of the *uidA* gene in the transgenic tissue. Oligonucleotides GUS forward 5′-GGTGGTCAGTCCCTTATGTTACG-3′ and GUS reverse 5′-CCGGCATAGTTAAAGAAATCATG-3′ were used as primers to check the specific amplification of *uid*A gene, which is in the size of 525 bp fragment.

RESULTS AND DISCUSSION

The success in tomato transformation could be largely affected by the genotype, type of explant and plant growth regulators used in culture mediums. In this study we optimized a repeatable method for the regeneration and transformation of three elite cultivars, which are widely used for commercial purposes in Iran. Hypocotyls and cotyledon explants of all three genotypes and mediums, mentioned in Table 1 and used in this experiment, were based on the results of other researches (Romero *et al*., 2001; Park *et al*., 2003; Velcheva *et al*., 2005).

Since the regeneration is quite dependent on the cultivar, as was mentioned in our literature review, therefore the regeneration of callus was done in the first step. The rate of callus production was different amongst different cultivars and media compositions. These media were classified based on the rate of callus induction. The maximum rate of callus induction in tested media was observed in M2 and M4 media with hypocotyls explant (Fig. 1). Present results showed that the application of

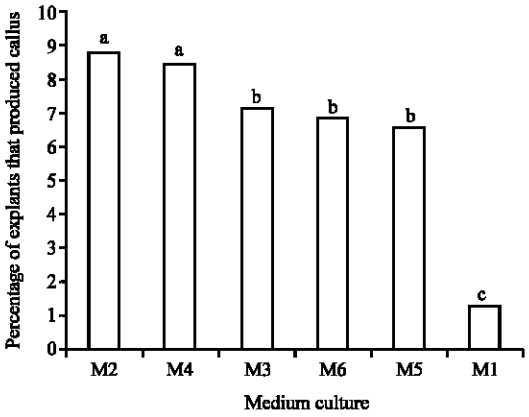

Fig. 1: Effect of medium on the percentage of explants that produced callus in four weeks time ($p<0.05$)

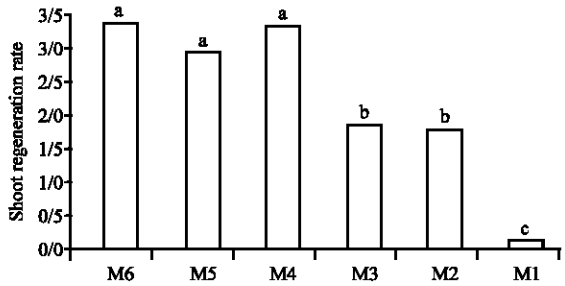

Fig. 2: Effect of medium on the percentage of explants that produced shoots ($p<0.05$)

callus for regeneration could not be suggested, mainly due to the somaclonal variation (Data not shown). These results are in accordance with that of Soniya *et al.* (2001), reported on tomatoes and the other solanacea family. Therefore, direct regeneration from transformed explants (hypocotyl and cotyledone) was used as a strategy. After four weeks, the mean number of shoots per each type of explant was scored and used as a regeneration efficiency for all three genotypes. Explants showed different responses in the presence of various growth regulators. The maximum shoot regeneration for cotyledons was observed on MS media, supplemented with 2 mg L^{-1} Zeatin with KalG genotype, but for hypocotyls, the high frequency of regeneration was observed in MS media containing 2 mg L^{-1} BAP m or 2 mg L^{-1} Zeatin combined with 0.1 mg L^{-1} NAA using Su2270 cultivar. The effect of 2 mg L^{-1} Zeatin was significantly different between the three genotypes ($p<0.05$) (Fig. 2). Many researches have previously reported that Zeatin stimulates the organogenesis of different tomato explants, such as leaves (Arillage *et al.*, 1998), cotyledon (Drorri and Altman, 2001; Costa *et al.*, 2000; Frary and Earle, 1996; Park *et al.*, 2003) and hypocotyls (Frary and Earle, 1996; Park *et al.*, 2003) which quite agree with our results obtained on BAP.

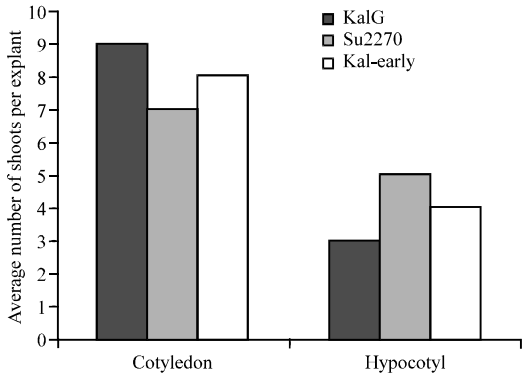

Fig. 3: Results of the average number of shoots per explants during *in vitro* regeneration of three cultivars

The optimal medium for shoot regeneration is quite dependent on cultivars as reported by few researches. For example Velcheva *et al.* (2005) reported that optimal regeneration of 2 cultivars was obtained on MS medium supplemented with 1 mg L^{-1} Zeatin and 0.5 mg L^{-1} IAA but for two other cultivars 2 mg L^{-1} Zeatin and 0.1 mg L^{-1} IAA were found to be optimal. Park *et al.* (2003) reported that the transformation frequency of five tested cultivars was greater on media containing 2 mg L^{-1} Zeatin and 0.1 mg L^{-1} IAA, indicating that there were no significant differences in the transformation frequency amongst explants or plant growth regulators tested. It seems that all steps of the regeneration and transformation process were cultivar-dependent.

Also, results of the analysis of variance showed that there are high significant differences among explants and genotypes. Among the three genotypes, the KalG and Kal-early showed the highest and lowest values of 30 and 10 for the percentage of explants producing shoots, respectively. The highest values for the average number of shoots per explants were observed on cotyledons KalG and hypocotyls Su2270 (9 and 5, respectively) (Fig. 3). Pozueta-Romero *et al.* (2001) observed that shoot regeneration per explant were different amongst tomato cultivars. Their experiments revealed that the ability of organogenic ranged from 2.8 shoots per explant in Rutgers to 5.3 in UC828 cultivars. Comparing of the mean number of shoots per explants, in transformed and control explants, indicated that shoot formation and regeneration efficiency were critically reduced, which supports the results obtained by others (Park *et al.*, 2003; Velcheva *et al.*, 2005; Hamza and Chupeau, 1993; Costa *et al.*, 2000).

Our results also showed that the application of hormones in media for the achievement of optimal shoot elongation and rooting is not apparently so critical (Table 2). On an average, 50% of the shoots were elongated after transfer to (EM) medium. On the other

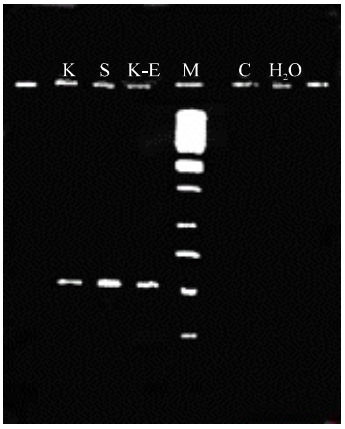

Fig. 4: PCR analysis for the presence of GUS gene in the regenerated plants. GUS gene specific primers were used that give a 525 bp fragment, K.KalG, S. Su2270, K-E. Kal-early, M. DNA standard 2500 bp lader, C. Non transformed regenerated plant. H_2O: Negative control

hand, there are a few reports which emphasized the use of growth regulators for elongation (Velcheva *et al*., 2005). It seems that in the hormone-free medium, explants can be elongated more easily, mainly due to the lack of the enforcing effect of hormones. But it could be also dependent on the cultivar, as we observed in the other steps of our experiment. Other researchers (Velcheva *et al*., 2005; Pozueta-Romero *et al*., 2001; Oktem *et al*., 1999; Lino *et al*., 2004) reported that the presence of IAA in the rooting medium is quite nessecary. Therefore it seems that root formation is also strongly genotype-dependent. More than 90% of putative transformed shoots were rooted on the MS basal medium which lacked growth regulators and supplemented with 100 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin (RTM). However, after two weeks, all control shoots died. After 4-6 weeks the plants produced enough roots and the growth of their plantlets were supported upon their transfer to the soil.

Molecular analysis and Histochemical *GUS* assay of transgenic plants: The presence of the *uidA* gene was analyzed by PCR. Agarose gel electrophoresis of amplified fragment (with designed primer for a part of gene) showed the presence of a 525 bp band related to the *gus* gene for each genotype (Fig. 4). Histochemical assay of explants revealed the *gus* expression after 2, 4 and 8 weeks of transformation experiments with both explants (Fig. 5). The percentage of each explants having *gus* activity was significantly different among these three cultivars. KalG and Kal-early cultivars showed the highest and lowest values with respect to the percentage of explants with *gus* positive spots (35 and 17%, respectively) (Fig. 6).

Fig. 5: GUS assay of tomato (a) transformed explants and (b) regenerated plants

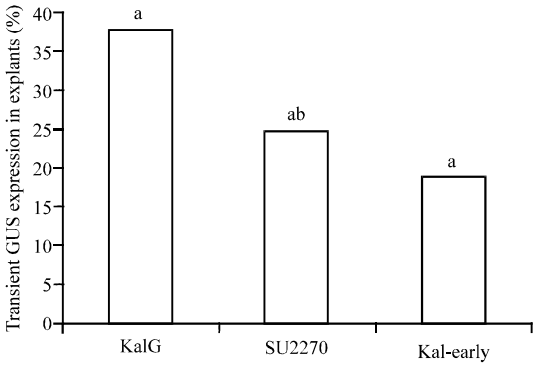

Fig. 6: Transformation efficiencies of tomato based on GUS expression ($p<0.05$)

Generally our results confirmed that the cotyledon is the best explant in our tomato transformation procedure.

The type of phytohormones seems to be a crucial factor controlling tomato regeneration. The effect of Zeatin on regeneration efficiency and transformation was more significant among the three genotypes when compared with other Plant Growth Regulators studied in these experiments ($p<0.05$), but considering its cost, BAP could be another choice.

Our results indicated that transformation and regeneration can be simplified in tomatoes (at least in KalG cultivar) by using Zeatin for regeneration and hormone-free MS medium for elongation and rooting.

Taken together, it seems that all aspects of transformation and regeneration were cultivar-dependent. Results of this study revealed that KalG was the best genotype for the regeneration and transformation efficiency and could be used in further research for other purposes like resistance to disease or production of pharmaceuticals.

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