PROGRESS IN BIOLOGICAL SCIENCES

Regeneration and *Agrobacterium*-mediated transformation of three economically important strawberry cultivars Kurdistan, Camarosa and Paros

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Abstract

Genetic transformation studies were carried out to standardize a protocol for *Agrobacterium*-mediated genetic transformation of three economically important strawberry (*Fragaria* x *ananassa* Duch) cultivars Kurdistan', Camarosa', and 'Paros'. Shoot regeneration frequency 72, 65 and 30% was obtained on MS (1) basal medium supplemented with 2% glucose and 4 mg/l TDZ for Camarosa, Kurdistan and Paros, respectively. To optimize the concentration of kanamycin for these cultivars, we observed the responses of leaf explants to different concentration of kanamycin (0-100 mg/l) in the media. Our results showed that 75 mg/l was an effective concentration of kanamycin. For genetic transformation, *Agrobacterium tumefaciens* strain C58C1RifR (pGV2260) harbouring the binary vector pTJK136 containing *uidA* gene with intron along with kanamycin resistance gene (*npt-II*) was used. After five days pre-incubation and 72 h co-cultivation, transformed cells (explants) were able to grow on the selective regeneration medium containing 75 mg/l kanamycin and 500 mg/l cefotaxime, while, control explants failed to grow. The regenerated putative transgenic shoots were analyzed by histochemical GUS assay and PCR analysis. Transformation efficiency based on inoculated explants number was 2, 1 and 3% for ', Camarosa', 'Paros ', and 'Kurdistan', respectively. The standardized protocol would be useful for *Agrobacterium*-mediated genetic transformation of these cultivars with important agronomic genes.

Keywords: *Agrobacterium tumifaciens*, Genetic transformation, GUS, strawberry

Introduction

Strawberries are flavorurful and nutritious fruit enjoyed by millions of people in all climates with an increasing demand. The berry is valued for its low-calorie carbohydrate, high fibber contents, a sources of natural antioxidants including carotenoids, vitamins, phenols, flavonoids, dietary glutathionine, and endogenous metabolites (2). About 65% of the Iran's strawberry is produced in Kurdistan province. The cultivars Ferzno, Misyoneri, Kurdistan, Merk, Selva, Camarosa, Paros, Qoein Eliza are under commercial cultivation in Kurdistan. The cultivar Kurdistan is the dominant cultivar followed by Camarosa and Paros.

Proper regeneration system is crucial to the successful application of *in vitro* methods. Genetic engineering of these valuable strawberry cultivars requires the development of transformation protocols and the establishment of efficient regeneration systems. Previous studies on strawberry have clearly demonstrated that various important factors such as genotype, source of explant, plant growth regulators and incubation time conditions significantly influence regeneration of strawberry (3, 4, 5). Genetic engineering of strawberry has already been reported; however, transformation frequencies are greatly influenced by genotype. Transformation frequency reported for strawberry cultivars has showed various ranges from 0.02 to 58%. For example, transformation efficiencies of 4.2% for the cultivar Chandler (6), 8-9.5% for Elista cultivar (4, 5) and 0.2% for Joliette cultivar (7) has been

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reported.
In this investigation, we have optimized a regeneration protocol for the strawberry cultivars Paros, Camarosa and Kurdistan; afterwards, we have attempted the transformation of the cultivar via *A. tumefaciens.* Using the leaf disk transformation method we examined a number of variables that may affect transformation of strawberry cultivars including choice and concentration of antibiotics in the selection medium; effect of preculture on the regeneration of kanamycin resistant shoots, effect of co-cultivation period and the genotype.

Materials and methods

Young leaf explants organ were collected from strawberry (*Fragaria ananassa*) plants cultivars of 'Kurdistan', 'Camarosa', and 'Paros' Explants were washed for 30 min in water tab and disinfected by commercial sodium hypochlorite solution (0.5% effective chlorine) for 15 min, then washed three times in sterile distilled water under aseptic conditions. Portions from leaves approximately 0.5 cm^2 were used as explants. The leaf segments were preincubated for a week in MS medium supplemented with 4 mg/l TDZ and 2% glucose in dark condition.

Transformation and regeneration

A. tumefaciens strain C58C1RifR (pGV2260) harboring the binary vector pTJK136 containing *uidA* gene with intron **(**8) was used in the genetic transformation experiments. This plasmid contains left and right borders of the transfer region of Ti plasmid, the selection gene *nptII* (neomycin phosphotransferase II) that confers kanamycin resistancein plants, and the reporter gene *uidA* (GUS), both undercontrol of the CaMV 35S (Cauliflower mosaic virus 35S) constitutive promoter. The intron inserted in the protein-coding region of gus fragment blocks the expression of this gene in *A. tumefaciens*.
For inoculum preparation, one bacterial colony was transferred to LB liquid medium supplemented with kanamycin (50 mg/l) and rifampicin (20 mg/l) and incubated for 16 h on an orbital shaker (28°C/180 rpm). When the culture was at density of OD600 = 1 , cells were pelleted by centrifugation at room temperature, 5000 rpm for 10 min, and the resulting pellet was resuspended in 25 ml liquid MS medium and acetosyringon was added (100 μM).

Sensitivity test of strawberry explants to kanamycin

The sensitivity of the explants to kanamycin was examined by culturing the leaves without co-cultivation with *A. Tumefaciens* on selection medium containing different concentrations of kanamycin (0, 50, 75 mg/l). For genetic transformation of explants were precultured on MS medium supplemented with glucose (2%), 4 mg/l TDZ, agar (7 g/l), and the pH was adjusted to 5.8 (Fig. 1a). Afterwards, explants were inoculated with the bacterial suspension for 20 min and then dried on sterile filter paper to remove excess of bacteria. The material was incubated (co-cultivated) for three days in darkness at 23°C in MS medium without the selection antibiotics. After co-cultivation, the explants were washed with sterile distillated water for five min and in 800 mg/l cefotaxime solution for 15 min and dried on sterile filter paper. Then explants were transferred to the medium with cefotaxime 800 mg/l for five days.
Then explants were then transferred to selection medium [MS medium supplemented with glucose (2%), 4 mg/l TDZ agar (7 g/l), kanamycin (75 mg/l), sodium cefotaxime (500 mg/l), pH 5.8]. After 5 weeks, explants were transferred to the second selection medium [MS supplemented with glucose (1%), sucrose (2%), agar (7 g/l), BA 0.2 mg/l, GA3 2 mg/l kanamycin (50 mg/l), sodium cefotaxime (500 mg/l), and pH 5.8] for 4 weeks.

Histochemical gus assay

Kanamycin-resistant regenerated plantlets were immersed overnight in a reaction mixture containing X gluc as substrate, and gus activity was analyzed histochemically as described by Jefferson (9). After 24 hours, the material was washed with ethanol (70%) to remove chlorophyll to easily access the blue color reaction in the transformed tissue.

PCR analysis

DNA was extracted using CTAB method with little variation (10). Approximately 5 g of leaf tissue was

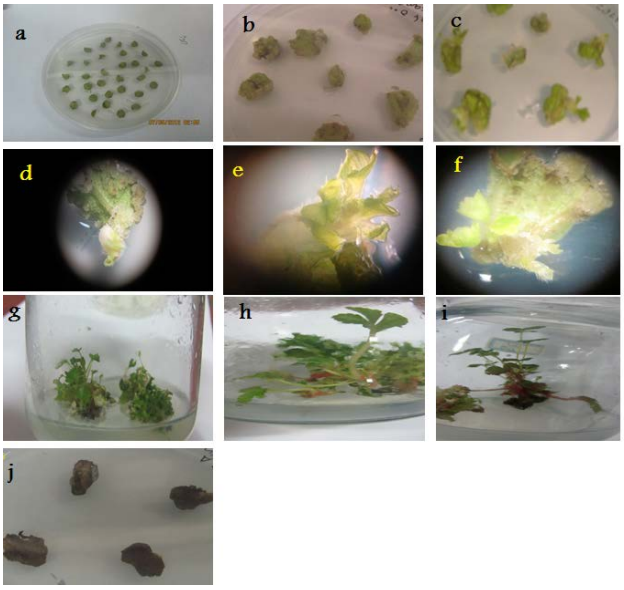

Figure 1. Genetic transformation and regeneration of three cultivars of strawberry using leaf disc method. **(a)** Explants in regeneration medium before co-culture. **(b)** Early callus development in 'Paros' leaf disc region 15 days after co-culture in regeneration medium in darkness**. (c, d)** Early developed shoot via indirect organogenesis, in 'Camarosa' explants, 30 days after co-culture in regeneration medium (selection medium) in darkness. **(e, f))** Differentiation of shoot after co-culture in second selection medium in darkness. **(g, h)** Multiple shoot formation **(i)** Transgenic plantlets (**j**) Leaf discs that were not transformed on kanamycin 75 mg/l.

collected from leaves and well crushed after adding liquid nitrogen in a mortar with pestle. Then 1 ml of extraction buffer (4% CTAB, 20 mM EDTA, Tris 100 mM, 2% PVP-10, pH: 8.0) was added and stirred until well mixed. Subsequently, pour in a 2 ml microtube, add approximately 10 µl mercaptoethanol, and kept in water bath for 45 minutes at 65°C (every 10 minutes will slowly invert). DNA was dissolved in 50 µL of TE buffer and stored at -20ºC.

Quality and quantity of the extracted DNA were checked by agrose gel and NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The PCR reaction was carried out with 100 ng of genomic DNA, 0.5 µL dNTPs (10 mM), 0.7 µL $MgCl_2$ (25 mM), 2.0 µL of buffer, 0.2 µLTaq polymerase (5 U lL-1), and 1 µL of each primer (10 mM). For *gus* gene amplification, the primers GUS-156F (5′-CGATCAGGTCGCCGAGG-3′) and GUS-1560R (5′-GGTCGCGGAGGGTATCG-3′) were used, with the following reaction program: 94°C for 4 min; 35 cycles of 94°C for 3′, 65°C for 60 s and 72°C for 60 s, and to finalize 72°C for 5 min, amplifying a fragment of 1406 bp. Wild type strawberry (not infected with *A. tumifaciens*) DNA was used as a negative control and the plasmid pTJK136 was used as a positive control. Amplified DNA fragments were electrophoresed on agarose gel (1.2%) containing ethidium bromide (0.5 µg/ml) and visualized and photographed under UV light (Fig. 3).

Results

Regeneration

Shoot regeneration of strawberry cultivars depends on media composition, leaf physiology and genotype. Based on the percentage of explants displaying shoot formation, MS media supplemented with 2% glucose and 4 mg/l TDZ was the most suitable for the strawberry cultivars Paros, Camarosa and Kurdistan indicating a regeneration percentage of 72, 65 and 30 percent after five weeks, respectively.

Influence of kanamycin on shoot regeneration from leaf explants

After five weeks, 65, 33 and 72% callus induction was attained in explants cultured on the callus induction medium lacking kanamycin for Camarosa, Kurdistan and Paros, respectively. On the medium containing 50 mg/l kanamycin, the percentage of explants with at least one or more shoots was 5, 3 and 6% for Camarosa, Kurdistan and Paros, respectively (Table 1). At 75 mg/l 100% of explants for all three cultivars bleached and died (Fig. 1j). Based on these results to minimize escapes and prevent necrosis, we chose 75 mg/l kanamycin for the transformation experiments (Table 1).

Transformation of strawberry cultivars

Leaf disc of three cultivars Paros, Camarosa and Kurdistan were transformed using *A. tumefaciens* strain C58C1RifR (pGV2260) harboring the binary vector pTJK136 containing *uidA* gene with intron **(**8). Explants were initially transferred to shoot

Table 1. Effect of kanamycin concentration on regeneration potential of Camarosa, Paros and Kurdistan leaf explants.

Regeneration explants (%) After 5 weeks			
Paros	**Kurdistan**	**Camarosa**	**Kanamycin(mg/l)**
72	33	65	0
6	3	5	50
0	0	0	75
0	0	0	100

induction medium in which an average of 17, 14 and 10 shoots were generated from each leaf explants in Paros, Camarosa and Kurdistan, respectively (Table 2). Explants were co-cultivated for a period of 48 and 72 h in dark. The Leaf disks with 48 h co-cultivation failed to grow after five weeks on co-cultivation medium, while leaf disks with 72 h co-cultivation were regenerated efficiently. After co-cultivation, the leaf explants were washed with milliQ water twice (5 min each) and then with cefotaxime (800 mg/l) solution for 15 min. The explants were then transferred to pre-selection regeneration medium for five days with cefotaxime (500 mg/l). After pre-selection, the explants were transferred to fresh selective regeneration media for 5 weeks with 75 mg/l kanamycin for the selection of transformed cells and to inhibit further agro-bacterial growth (Fig. 1b, c, d). After five weeks, while most uninfected explants died, leaf explants with kanamycin-resistant shoots were regenerated (Fig. 1e-i).

Table 2. Genetic transformation efficiency of three strawberry cultivars from explants of adult plants.

Cultivar	Inoculated explants	Regeneration of transformants on kanamycin(%)	GUS+PCR+ explants	Transformation Efficiency (%)
Camarosa	100	14	2	2
Paros	100	17	3	3
Kurdistan	100	10	1	1

Analysis of transgenic plants

The confirmation of genetic transformation was done by GUS histochemical analysis on regenerated shoots, and by PCR to confirm the insertion of the *uidA* reporter gene. Staining of transgenic plants disclosed that the reorter gene is expressed in all tissues (Fig. 2). Since the GUS-INT construct was used, we can safely conclude that the blue histochemical colour is due to the actual integration and expression of this gene after splicing and not due to *Agrobacterium* contamination. The control of leaf callus did not show any blue coloration (Fig. 2).

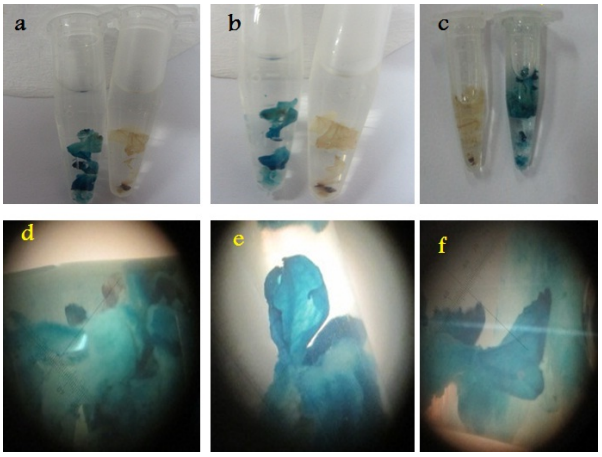

Figure. 2. Expression patterns of the *uidA* gene in the putatively transformed plantlets. **(a, b and c)** *GUS* expression in transgenic leaf compared to non transgenic control of Kurdistan, Comarosa and Paros, respectively. **(d)** Kurdistan regenerated plantlet in selection medium and incubated in X-Gluc substrate showing the expression of uidA gene. **(e)** Camarosa regenerated plantlet in selection medium and incubated in X-Gluc substrate. **(f)** Paros regenerated plantlet in selection medium and incubated with X-Gluc substrate.

To confirm transformation event at molecular level, total DNA was extracted and analysed from the leaves of both non-transformed and transgenic plants (Fig. 3). Putative transgenic plants were screened by PCR using gus gene-specific primers to detect the presence of the transgene. PCR

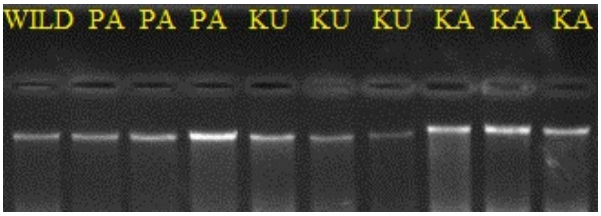

Figure. 3. Genomic DNA extraction of putative transgenic strawberry plants and wild type plant. WILD: DNA from wild type strawberry. PA: DNA from Paros transgenic shoots. KU: DNA from Kurdistan transgenic shoots. KA: DNA from Camarosa transgenic shoots.

analysis showed the amplification of the predicted 1406 bp fragment in kanamycin-resistant

transformed plants for all three cultivars (Fig. 4). No amplification product was detected in the negative control (Fig. 4 lane N), while positive plasmid control produced the same bands as transgenic plants. (Fig. 4 lane P).

Transformation efficiency was evaluated as the number of GUS and PCR positive plants per total number of inoculated explants. On average, contamination rates were 14, 17, and 10% for ‘, Camarosa’, ‘Paros ’, and ‘Kurdistan’, respectively. Transformation efficiency based on inoculated explants number was 2, 1 and 3% for ‘, Camarosa’, ‘Paros ’, and ‘Kurdistan’, respectively (Table 2).

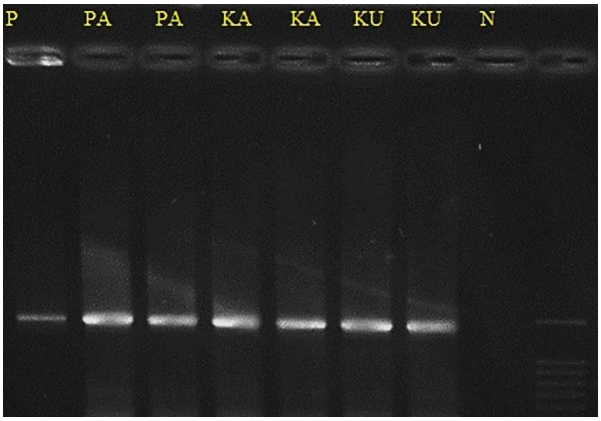

Figure 4. Polymerase chain reaction analysis of three cultivars of strawberry selected transgenic lines showing integration of gus gene (1406bp) in the genome of selected transformed plants. PA: PCR of Paros transgenic shoots. KU: PCR of Kurdistan transgenic shoots. KA: PCR of Camarosa transgenic shoots. N: negative control (Non-transgenic strawberry plants). P: positive control DNA from plasmid.

Discussion

In this study, we present a protocol for efficient and stable transformation of three economically important strawberry cultivars in Kurdistan province of Iran. Explants were cultured on the regeneration medium, (MS; 2% Glucose, 4 mg/l TDZ), which resulted in high regeneration rate after five weeks especially for Paros and Camarosa. Robust regeneration system based on thidiazuron (TDZ) via shoot organogenesis has been described in different cultivars of strawberry (11, 12, 13, 14, 15, 16, 17). TDZ is a synthetic growth regulator that shows the property of both auxin and cytokinin (18). Significant positive effect of TDZ on shoot regeneration from leaf disks of strawberry cultivar ‘Chandler’ has been reported (17). Using 4 mg/l TDZ they achieved up to 85% regeneration rate after eight weeks. Furthermore, utilization of TDZ in diploid (19) and octoploid strawberry (19, 20, 21) has shown to have positive effects on shoot proliferation and finally higher likelihood of obtaining transgenic shoots (16). Thus, similar to previous works, TDZ had positive effect on strawberry varieties Paros, Camarosa and Kurdistan during regeneration.

The influences of different concentration of kanamycin on shoot regeneration of three strawberry cultivars were studied. At 75 mg/l all explants were dead after 5 weeks, so this concentration was chosen to select transformed plants. Shoot regeneration from leaf disks is impaired at kanamycin concentrations as low as 10 mg/l, but a concentration of 25-50 mg/l is generally employed to avoid the risk of recovering non-transgenic shoots (4, 6, 22). Mathews et al. (23, 24) found a high percentage of chimerism in regenerated shoots of the strawberry cultivar Totem, using continuous selection on 25 mg/l kanamycin. Schaart et al. (11) applied 75 mg/l kanamycin on young leaf discs of strawberry cultivar Garigutte. Schestibratov and Dolgov (25) found a high percentage of regenerated transgenic shoots of strawberry cultivar Firework using 50 mg/l kanamycin. Furthermore, Gruchała et al. (2004) (4 or 5) compared different concentration of kanamycin, (0, 10, 25, 30 and 50 mg/l) on leaf explants cultivars Elista and Induka and showed that 25 mg/l for ‘Elista’ and 30 mg/l for ‘Induka’ is selective. These researches showed that the concentration of kanamycin in selective medium depends on strawberry genotypes.

Transformation efficiency based on inoculated explants number was 2, 3 and 1% for Camarosa, Paros, and Kurdistan, respectively. The transformation efficiency was relatively low. Transformation efficiency is typically described as the percentage of explants that produce a transgenic shoots. Previous transformation studies on strawberry cultivars resulted in wide range of transformation rate from 0.02 to 58%. Transformation efficiency for cultivars ‘Rapella’ (26), ‘Redcoat’ (27), and ‘Rhapsody’ (28) were 0.02, 0.4 and 0.6%, respectively, while for ‘Totem’ (22) was as high as 58%. In another study,

transformation percentages for cultivars 'Symphony' and 'SengaSengana' (29) were 14.2 and 2.7%, respectively. In addition to difference in transformation rate among genotypes, based on transformation methods, wide variation in transformation percentage for specific cultivars has been reported. For example, using *Agrobacterium*-mediated transformation of strawberry cultivar Chandler resulted in efficiency of 4.22% (6), while combining *Agrobacterium* infection and biolistic bombardment, leaf discs transformation rate 22.7% was reported (30). Thus, difference in transformation efficiency can also be phenomenon is related to genotype and regeneration ability on selection medium.

In the present study, Agro-inoculation for 20 min, followed by co-cultivation for 72 h resulted in best transformation percentage. During co-cultivation, increasing concentration of bacteria impose stress on plant cells and reduce their regeneration potential. Meanwhile reducing number of bacteria results in reduction the frequency of T-DNA transfer (31). In addition to bacterial concentration, period of co-cultivation is another important factor. Increased co-cultivation period can increase transformation rate, but may cause tissue necrosis and dying explant (32). Zhang and Wang (33) in a similar research to our study, discovered that the optimal conditions for *Agrobacterium*-mediated transformation of cultivar 'Allstar' leaf disks was inoculation for 10-15 min followed by co-cultivation for three days. Using this condition, they achieved a transformation percentage of 1.1% based on kanamycin resistance assays.

In the present study, 7-day pre-culture was found beneficial and improved average transformation percentage. Pre-culture (pre-incubation) of explants in proper conditions prior to *Agrobacterium* inoculation can improve transformation rate by increasing the number of cells competent for regeneration (34). Diverse pre-incubation time periods from 3 to 10 days for different explants of different strawberry cultivars to improve regeneration rate has been reported (6, 30, 35). Asao et al. (36), cultured leaf and petiole explants of strawberry for one day in liquid medium prior to *Agrobacterium* inoculation.

In our report, we present a protocol for efficient and stable introduction of *gus* gene into three economically strawberry cultivars. Evidence for highly efficient transformation was obtained by PCR and GUS staining analyses. The standardized protocol would be useful for *Agrobacterium*-mediated genetic transformation of these cultivars with genes with agronomic importance.

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