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Assessment of Conditions Influencing *Agrobacterium*-mediated Transient Expression of *uidA* Gene in Leaf Disks of Sugarbeet (*Beta vulgaris*)

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Optimum Agrobacterium-mediated transformation condition on sugarbeet (ELK345) leaf disks were examined in this study. Parameters are "vacuum infiltration, different bacterial growth medium, inoculation time with bacteria, treatment bacterial strains (EHA105, LBA4404 and GV2260) and L-cysteine in co-cultivation medium" that were studied for optimization. After 3 days, effect of these parameters on transformation was evaluated by histochemical GUS assays. Leaf disks on blue spots were quantitatively analyzed by Zeiss® KS300 image analysis platform. Transient gene expression was enhanced upto 3 fold both by vacuum infiltration at 400 mmHg and Agrobacterium strain GV2260. Selection schemes using phosphinotricin or kanamycin as selective agents were also assessed. Medium containing 150 mg/l kanamycin or 3 mg/L phosphinotricin (PPT) totally inhibited the shoot regeneration. These transformation and selection procedure may be amenable to genetic transformation and may provide a valuable tool in sugarbeet improvement programs.

Keywords: ***Agrobacterium strains, Beta vulgaris, kanamycin, phosphinotricin, sugarbeet, transient uidA gene expression, vacuum infiltration.***

Introduction

Sugarbeet is a sugar producing crop of economically important in Europe and other temperate regions of the world. Approximately 30 % of the world's sugar is produced from sugarbeet. Although over the past several decades much effort has been given to improving regeneration and transformation techniques for this crop, there are some difficulties both in tissue culture and transformation. Difficulties such as strong genotype dependence, somaclonal variation and low reproducibility are described in several reports [1,2]. Early attempts to produce transgenic sugarbeet using *Agrobacterium* met with only limited success. Krens [3] obtained transformed calli via *Agrobacterium*-mediated transformation;

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however, they could not produce regenerated transformants. Lindsey and Gallois [4] first reported the production of transformed sugarbeet plants from shoot base tissue slices via *A. tumefaciens* infection. However, only few of resistant shoots exhibited screenable gene activity in their study. D'Halluin [5] produced transgenic sugarbeets via callus regeneration using tobacco suspension cells as a nutrient layer but it was an impractical study due to initiation of transgenic shoots from suspension culture. Konwar [6] also showed that shoot bases can be infected with *A. tumefaciens* to produce transgenic plants. Snyder [7] developed two different methods for sugarbeet transformation, one is using *Agrobacterium* with excised cotyledons, and the other is particle bombardment of embryogenic hypocotyl callus. However, transformation was restricted to only one genotype. The most recent studies on sugarbeet transformation are development of a new *Agrobacterium*-mediated gene transfer method using shoot base of sugarbeet [8], production of sugarbeet plants with improved salt-tolerance [9] and production of transgenetic sugarbeet plants resistant to phosphinothricin [10].

Although sugarbeet is susceptible to infection by *A. tumefaciens* [11], transformation efficiency is very low. There is little information about factors that influence genetic transformation. There are also some reports which indicate transient gene expression in sugarbeet using *Agrobacterium*-mediated transformation or particle bombardment to examine effect of various factors on transformation of sugarbeet. Jacq [12] showed that several factors including genotypes, acetosyringone, coculture and preculture durations affect transformation efficiency of *Agrobacterium*-mediated transformation. Mahn [11] used the apices of sugarbeet seedlings as targets for particle bombardment but the rate of transient expression was very low. Önde [13] transferred a ß-Glucuronidase reporter gene to sugarbeet callus and leaf explants via microprojectile bombardment. For evaluation of GUS positive regions, blue spots are counted in bombardment reports. This method is extremely time-consuming and Jacq [12] indicated transformation frequency by the percentage of seedling explants showing GUS activity. These methods have a high error rate.

Despite these promising results, *Agrobacterium*-mediated transformation methodologies in sugarbeet should be improved due its widespread use in vast number of transformation laboratories. In this study, we have, for the first time, investigated effect of various parameters (vacuum infiltration, bacterial growth medium, inoculation time with bacteria, type of the bacterial strain and the addition of L-cysteine in co-cultivation medium) on transient GUS gene expression using in leaf disk explants. To our knowledge this is the first comprehensive work to evaluate *Agrobacterium* transformation capacity of leaf disks of sugarbeet.

Material and Methods

Preparation of plant material

The sugar beet cultivar ELK 315 commonly cultivated in Turkey was used in the experiments. Seeds were kindly provided by the Sugar Institute, in Ankara. The seeds were surface- sterilized in 70 % ethanol for 5 minutes, in 80 % sodium hypochlorite for one hour by continuous stirring with magnetic stirrer and washed three times with sdH_2O. Seeds were kept in sdH_2O overnight in dark at 23°C for imbibition. Imbibed seeds were rinsed in 5 % PPM™ solution (Plant Preservation Mixture, Plant Cell Technology Inc. WA, USA) for 10 minutes. Seeds were germinated on MS medium [14] containing 3 % sucrose and 0.8 % agar at 24 ± 2°C under light with a 16/8 hour (light/dark) photoperiod. For determination of appropriate conditions for sugarbeet transformation, 10-15 days old sugarbeet leaves were used in transformation studies.

Preparation of Agrobacterium cells

A single colony of *A. tumefaciens* strains (EHA105, GV2260 and LBA4404 harboring the binary plasmid pGUSINT) were grown overnight at 28 ± 1°C with 180-200 rpm shaking incubator in 5 ml liquid YEB medium supplemented with appropriate antibiotics. Then 500 mL of liquid YEB medium was inoculated with 100 μL of the overnight grown initial culture. The bacterial culture was grown overnight at 28±1° C at 180-200 rpm till optical density (OD_{600}) reaches to 0.8 value. Then the culture was centrifuged at 1500 g for 15 minutes at 4°C. The pellet was resuspended with inoculation medium (Sucrose 20 g, MS salts 4.3 g, MES 1.95 g pH: 5.6) to final OD_{600} value of 2.4. Finally the bacterial suspension was incubated at 24±2°C under dark conditions for 1 hour and then used for transformation.

Vacuum infiltration

For transformation of leaf disks, 10-15 days old leaves of sugar beet were cut into small pieces from stock material. The explants were immersed in bactreia culture and then they were vacuum infiltrated at different pressures of 0, 200, 400 and 600 mmHg for 10 minutes.

Bacterial growth medium

A. tumefacien strains were grown overnight in three different standardized bacterial growth media; (i) YEB (Nutrient broth 13.5g, Yeast extract 1g, Sucrose 5g, $MgSO_4.7(H_2O)$ 0.493 g pH: 7.2), (ii) YEB+MES (Nutrient broth 13.5g, Yeast extract 1g, Sucrose 5g, $MgSO_4.7(H_2O)$ 0.493 g MES 2.132g, Acetosyringone 20 μM pH: 5.6) and (iii) MG/L [(Yeast extract 2.5 g, Mannitol 5g, Glutamic Acid 1g, KH_2PO_4 0.25 g, NaCl 0.25 g, Tryptone 5 g, $MgSO_4.7(H_2O)$ 0.1 g Biotin 1μg pH:7.0)] [15] supplemented with appropriate antibiotics. After transformation, they were directly transferred into different co-cultivation media called MS(MS salts 4.3 g, Sucrose 30 g, Plant Agar 4 g pH: 5.8) and MMD medium (MS salts 4.3 g, MES 1.95 g, Sucrose 15 g Phytagel 0.28 g. After autoclaving: 2,4-D 1 mg, Acetosyringone 200 μM, Ascorbic acid 100 mg pH: 5.6 were added).

Infection period

After the most appropirate vacuum pressure and the most suitable bacterial media were determined, leaf disks were inoculated with bacterial culture for different periods including 10, 20, 40 and 60 minutes in dark at 26°C.

Bacterial strains

For determination of effect of bacterial strains on transformation efficiency, different *Agrobacterium* strains including EHA105, GV2260 and LBA4404 were employed for transformation of leaf disks. Each strain contain binary plasmid pGUSINT carrying *uidA* (GUS) gene with Cauliflower Mosaic Virus 35S Promoter (CaMV35S promoter) and neomycin-phosphotransferase-II (*npt*-II) gene with nopaline synthase (nos) promoter and nopaline synthase terminator. Leaf disks were inoculated with these bacterial strains.

L-cysteine application

Various concentrations of L-cysteine (100, 200, 400, 800 and 1200 mg/L) were added to the cocultivation medium. Leaf disks were co-cultivated with the *Agrobacterium* suspension and maintained in cocultivation medium supplemented with these concentrations of L-cysteine.

Determination of selection conditions

Leaves of sugar beet were exposed to selective agents to determine their effect on direct shoot formation and to find out the lethal dose that can be used during transformation studies of sugar beet. Different combinations of kanamycin and PPT were employed for this purpose. Explants were cultured on medium containing selective agents and 0.1 IBA mg/L, 0.25 BA mg/L for 4 weeks. Number of shoots formed per leaf was recorded.

GUS histochemical assay

GUS histochemical staining was performed as described by Jefferson [16] using 1 mM X-Glucoronide dissolved in 1 mL dimethyl formamide. GUS expressing regions on explants were examined under microscope. For each treatment, leaf disks were photographed and analyzed by image analysis system (Zeiss® KS3000 in METU Central Laboratory). Percentage of GUS staining area for each leaf disk was calculated.

Statistical analysis

Each parameter was tested in 6 sets of 2 plates each containing 20 explants. Minitab 13.0 software was used for all of the statistical analyses. Means and standard error of means (SEM) were calculated by using this software. Oneway analysis of variance (ANOVA) was used to detect variances in terms of GUS expression units on explants which were exposed to different experimental treatments.

Results

Effect of vacuum infiltration

In order to indicate effect of vacuum infiltration on transformation efficiency; 200, 400 and 600 mmHg evacuation pressures were applied to leaves of sugarbeet for 10 minutes. The experiments were compared to control groups, which were not inoculated with bacteria and not infiltrated. Effect of infiltration represented as percent of explants exhibibting GUS activity on 3rd day after transformation is shown in Figure 1. In the control group, no GUS activity was obsreved. On the other hand, percentage of explants exhibiting GUS activity was increased when the explants were infiltrated at 400 mmHg (2.8 ± 0.3) compared to no vacuum applied explants (1.9 ± 0.2).

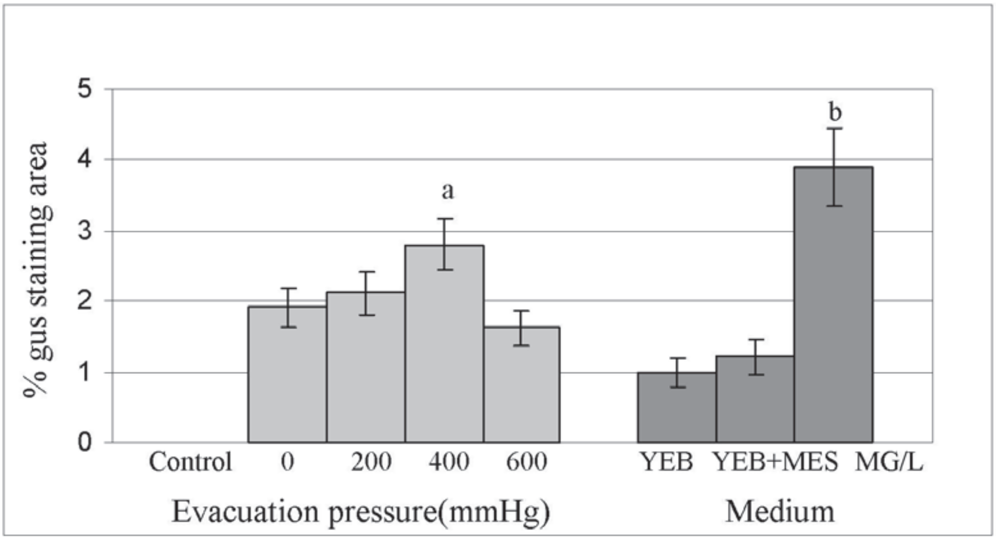

Fig. 1. Effect of vacuum infiltration and bacterial growth medium on transient gene expression 3 days after transformation. Vertical bars indicate SEM. Bars topped with different letters show significant differences among each treatments ($p < 0.05$).

Application of 400 mmHg evacuation pressure significantly increased the transformation efficiency in leaves of sugar beet according to GUS staining.

Effect of bacterial growth medium

In this study YEB, YEB+MES and MG/L medium were used for bacterial growth to examine their effect on transformation efficiency. Bacterial culture grown in these media was inoculated with leaf disks for 10 minutes at 400mmHg evacuation pressure.

Results of GUS histochemical staining described as percent GUS expressing area are displayed in Figure 1. Control explants, not inoculated with bacteria, exhibited no GUS activity. When YEB and YEB+MES media were used for transformation; 1.0 ± 0.2 and 1.2 ± 0.2 % of explants exhibited GUS activity, respectively. On the other hand when the MG/L medium was employed for bacterial growth, 3.9 ± 0.5 percent of explants exhibited GUS activity, which was significantly higher than others. In the light of these results, utilization of MG/L medium for bacterial growth significantly increased the transformation efficiency in leaf disk explant of sugarbeet.

Effect of infection period

For investigation of infection period on transformation efficiency leaf disks were inoculated with bacterial culture grown in MG/L medium for different periods of time (10, 20, 40 and 60 minutes) at 400 mmHg evacuation pressure.

Figure 2 indicates the effect of infection period on transient GUS activity characterized as percent of explants. None of control explants showed GUS activity. GUS activity was proportionally increased, when the inoculation time with bacteria was increased. When the explants were inoculated with bacteria for 10 minutes, 2.6 ± 0.3 % of explants exhibited GUS activity. 10 minutes of infection period resulted with the lowest

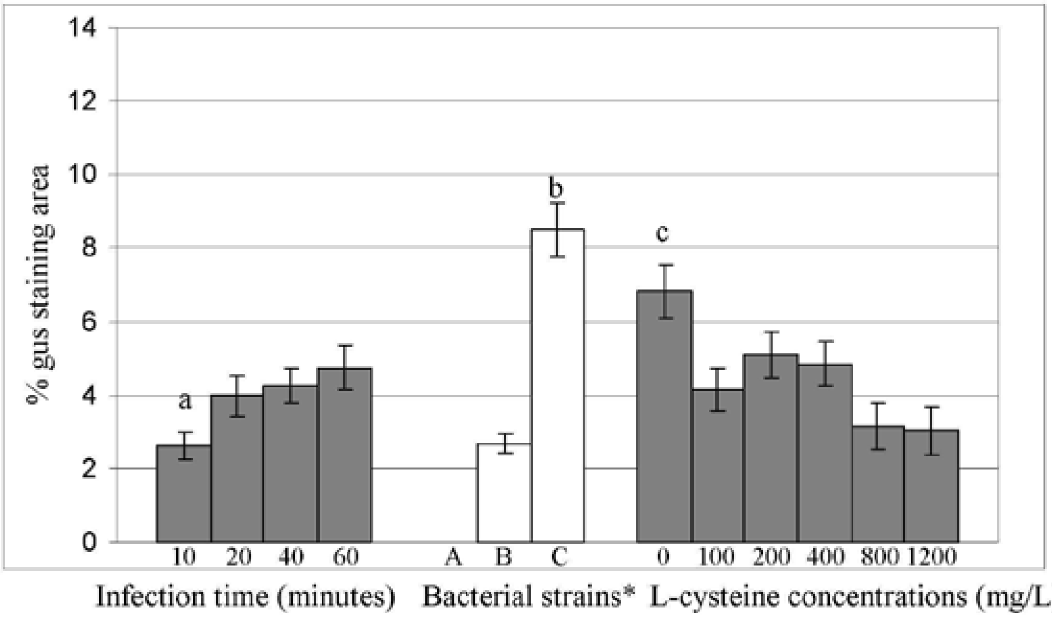

Fig. 2. Effect of infection period, *Agrobacterium* strains and different L-cyseine concentrations on transient gene expression 3 days after transformation. Vertical bars indicate SEM. Bars topped with different letters show significant differences among each treatments ($p< 0.05$) (Bacterial strains* A: LBA4404 B:EHA105 C:GV2260).

GUS activity. On the other hand, 20, 40 and 60 minutes of treatment with bacteria did not cause any significant ($p<0.05$) change in percentage of GUS expressing area which fluctuated between 3.9 and 4.7 %.

Effect of Agrobacterium strains

Efficiencies of three *Agrobacterium* strains EHA105, GV2260 and LBA4404 on transient GUS expression were compared. Leaf disks were inoculated with these strains grown in MG/L medium. Infection was performed for 20 minutes, at evacuation pressure 400 mmHg. As a control group, leaf disks that were not treated with bacteria were directly placed on co-cultivation medium.

Effect of bacterial strains on transient gene expression defined as % GUS activity is given in Figure 2. Control explants exhibit no GUS activity. Also leaf disks which were inoculated with LBA4404::pGUSINT showed no GUS activity due to necrosis of explants after the first day of transformation. When transformation was performed using GV2260::pGUSINT, percentage of explants exhibiting GUS activity was 8.5 ± 0.7. This was the highest value in terms of percentage of GUS staining area per leaf compared to other applications and demonstrated a significant ($p<0.05$) enhancement in transformation efficiency when compared to other experimental sets.

Effect of L-cysteine concentration

Solid co-cultivation medium supplemented with different concentrations of L-cysteine (100, 200, 400, 800 and 1200 mg/L) were prepared to examine their effects on *uidA* (GUS) gene expression. Non-inoculated leaf disks were used as control and L-cysteine lacking co-cultivation media (0 mg/L) were used as control for L-cysteine effect. Transformation was performed using *Agrobacterium* strain GV2260 for 20 minutes at 400 mmHg evacuation pressure.

Results of histochemical GUS assays are shown in Figure 2. 6.8 ± 0.7 percent of explant exhibited GUS activity when explants were cultured on L-cysteine free media. On the other hand, all concentrations of L-cysteine application reduced the percentage of GUS expressing area, which ranged from 3.0 to 5.1 %. Moreover, a decline in GUS staining area was observed correspondingly when concentration of L-cysteine was increased after the 200 mg/L L-cysteine application.

Effect of selective agents

Different concentrations of kanamycin and PPT were used 50, 100, 150, 200, 250 mg/L and 1, 3, 5, 10 mg/L, respectively. All experiments were carried out together with controls. Selective agent free medium contain 0.1 mg/ L indole-3-butyric acid (IBA) and 0.25 mg/L benzylaminopurine (BA).

Explants produced shoots when they were cultured on kanamycin free medium and MS medium supplemented with 50 and 100 mg/L kanamycin. However there was no shoot development when explants were cultured on 150, 200 and 250 mg/L kanamycin. High concentrations (150 mg/L or more) of kanamycin inhibited shoot regeneration from leaf blades. PPT was another selection agent used in this study. Shoot regeneration was observed when explants were cultured on PPT free medium or medium containing 1 mg/L PPT. However PPT at concentrations above 3 mg/L totally prevented the shoot regeneration. Table 1 shows the effect of kanamycin and PPT on shoot formation.

Discussion

The aim of this study was to develop a transient expression system for intact leaves of sugarbeet which is a recalcitrant species for stable transformation. Previous transient expression systems developed for sugarbeet have been based on both particle bombardment [11,13] and *Agrobacterium* mediated transformation [12] but these studies did not

Table 1. Effect of kanamycin and PPT on shoot inhibition

Kanamycin			
Kananycin concentrations mg/L	# of leaf	No of shoot regeneration	% of shoot regeneration
0	20	10 ± 1.83	50
50	20	8 ± 1.83	40
100	20	5 ± 1.83	25
150	20	0	–
200	20	0	–
250	20	0	–
PPT			
PPT concentrations mg/L	# of leaf	No of shoot regeneration	% of shoot regeneration
0	20	8 ± 1.52	40
1	20	5 ± 1.52	25
3	20	0	–
5	20	0	–
10	20	0	–

serve as an efficient transformation system. Our system is comprised of evaluation of different parameters using image analysis system and provides effective and reliable transformation system for stable gene integration to sugarbeet.

Vacuum infiltration is an effective way of promoting close contact between bacterium and host plant cell. Kapila [17] performed a study which describes an efficient transient expression system for intact leaves of Phaseolus vulgaris, Phaseolus acutifolius, poplar, and tobacco using vacuum infiltration–based *Agrobacterium* transformation method and demonstrated that strong induction of bacteria before infiltration is important for the success of transient expression. Our findings were consistent with these studies, in which transient gene expression was proportionally increased with evacuation pressures except for 600 mmHg pressure. Mahmoudian [18] demonstrated that vacuum infiltration of *A. tumefaciens* suspensions containing lentil explants resulted in high levels of transient gene expressions. Kishchenko [10] also tried petioles for *Agrobacterium*-mediated transformation using vacuum infiltration but they could not produce transgenic sugarbeet shoots.

However, vacuum infiltration alone did not guarantee an efficient transformation as was observed from results in Fig 3a and 3b. Use of various bacterial growth media for transformation, which is a first study for sugarbeet, highly enhanced transient gene expression. Approximately 1.5 folds increase in transient gene expression was observed by using MG/L medium for bacterial growth. Composition of MG/L medium may increase expression of vir-inducing genes which result more expression of GUS gene in T-DNA region. MG/L media, [15] which is frequently used for monocot transformation, is completely different from YEB and YEB+MES. MG/L medium contains mannitol, glutamic acid, biotin and various salts including NaCl and KH_2PO_4. In conjunction to these results, we propose that

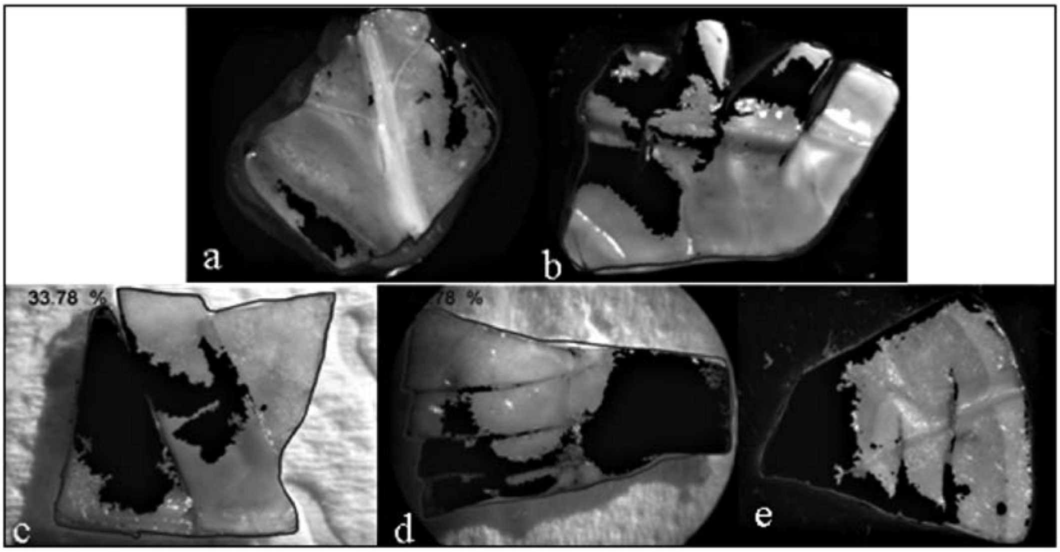

Fig. 3. Transient GUS expression of sugarbeet leaves after different application a) vacuum infiltration at 400 mmHg evacuation pressure b) bacterial growth medium in MG/L c) coculture period for 20 minutes d) *Agrobacterium* strain, GV2260 e) L-cysteine free media (Leaf disks were photographed and analyzed by image analysis system, Zeiss® KS3000)

vacuum infiltration is not a sufficient method for development of transient gene expression system for sugarbeet. Hence, these two parameters, usage of MG/L media caused induction of the vir genes and the application of induced bacteria to the plant tissue by infiltration, are responsible for an efficient system.

Infection period is another important factor affecting *Agrobacterium*-mediated transformation of beet. Successful transformation depends on attachment of suitable quantity of *Agrobacterium* on the cells. Yang [9] also examined the effect of infection and coculture period on beet transformation. They found that when the infection duration was short (1–3 min), few explants survived after selection. When the infection duration was higher than 20 min, *Agrobacterium* grew abundantly and majority of the explants necrotized. Our observations are similar with their findings. Although the percentage of transient GUS expression was increased in explants that were treated for 20 minutes, about half of explants showed necrosis formation. In contrast to other plants such as wheat [19], lentil [18] and bean [17], explants of sugar beet was generally incubated with bacteria for shorter periods. Thereby, necrosis formation and a decrease in regeneration capacity can be prevented. We propose that suitable infection period for beet transformation is 20 minutes (Fig 3c).

A significant ($p<0.05$) increase in transient gene expression level was observed when the GV2260::pGUSINT strain was used for beet transformation. This results in 3 folds increase in transient gene expression after the application of vacuum infiltration. When the efficiency of parameters are compared (Figure 3d), it is seen that the highest percentage of blue stained GUS expression area was monitored upon inoculation of explants with GV2260. LBA4404, EHA105, C58C1, KYRT1 and two binary plasmids, pGUSINT and pTJK136 were used for transformation of lentil. Our findings are consistent with this study, in which low gene expression frequency was observed by using EHA 105. Moreover, no GUS gene expression was observed when LBA4404 was employed for transformation. To our knowledge, these are the first results demonstrating the effect of different strains of *Agrobacterium* on transformation efficiency of sugarbeet. Utilization of the certain *Agrobacterium* strains can be useful for improvement of sugarbeet transformation system.

In *Agrobacterium* mediated transformation studies of rice and soybean, browning and necrosis of the plant tissues were observed due to wounding response. Tissue browning and necrosis may limit the success of transfer of T-DNA into viable cells [20]. The use of the L-cysteine, thiol compounds and antinecrotic compounds also resulted in increase in transformation efficiency in rice [21], soybean [20, 22] and grape [23]. We also investigated the effect of L-cysteine on *Agrobacterium*-mediated T-DNA delivery into sugarbeet leaves. In contrast to soybean and grape transformation, in which transformation efficiency was increased due to addition of L-cysteine to the solid co-cultivation medium, L-cysteine application did not cause any change in transient gene expression in our study. L-cysteine free cocultivation medium showed higher transient GUS expression than medium containing different concentrations of L-cysteine. Even a decrease in transient gene expression was observed after 200 mg/L L-cysteine application.

In order to select and recover the transformed cells or tissues from nontransformed ones, selectable marker genes including antibiotic resistance and herbicide tolerance are widely used [24]. They allow transformed cells expressing themselves to be selected over non-transformed cells. In our study, leaf blades from which shoot regeneration occured were exposed to different selective agents and could be efficiently suppressed on medium containing 150 mg/L kanamycin or more and 3 mg/L or more PPT. Color loss and necrosis was observed due to high concentrations of selective agents. This result is consistent with the report of Hisano [8], in which 150 mg/L kanamycin was preferred for selection of transgenic sugarbeet.

In conclusion we describe an *Agrobacterium*-mediated transient gene expression protocol that uses a combination of a different parameters and has a high potential to support rapid establishment of *Agrobacterium*-mediated sugarbeet transformation. Although GUS assays only exhibit the transient expression of the introduced gene, this protocol allows the improvement of *Agrobacterium* infection and production of desirable transgenic sugarbeet plants.

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