International Journal of Histology and Cytology ISSN 2756-3707 Vol. 7 (2), pp. 001-007, February, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Analysis and optimization of DNA delivery into chickpea (*Cicer arietinum* L.) seedlings by *Agrobacterium tumefacience*

Mikail Akbulut[1]*, Meral Yücel[2], Hüseyin Avni Öktem[2]

[1]Department of Biology, Erciyes University, 38039, Kayseri, Turkey. [2]Department of Biology, Plant Biotechnology R&D Laboratories, Middle East Technical University, 06531 Ankara, Turkey.

Accepted 17 November, 2019

The main purpose of this study was to develop a non-tissue culture based *Agrobacterium* mediated transformation method for chickpea. The influences of several factors were investigated on the transfer of β-glucuronidase (GUS) gene into chickpea (*Cicer arietinum*) seedlings during the early stages of *Agrobacterium*-mediated gene transfer, including cocultivation period in liquid induction medium (2, 8, 16 and 24 h), strains of *Agrobacterium tumefaciens* (C58C1, EHA105, KYRT1) containing the plasmid pTJK136, developmental stage (16 h imbibed and 40 h germinated), microwounding, vacuum infiltration (200, 400, 600 mmHg for 20 and 40 min) and genotype (5 different). The number of GUS-expressing foci was counted to evaluate the gene transfer process. The KYRT1/pTJK136 strain of *A. tumefaciens* was significantly more effective for transformation than the C58C1/pTJK136 and EHA105/pTJK136 strains. The highest transient GUS activity was obtained from 16 h imbibed seedlings of cv.Uzunlu wounded with a needle and co-cultivated in liquid induction medium for 24 h with the KYRT1 strain (226 GUS foci/per explant).

Key words: Chickpea, transformation, *Agrobacterium,* vacuum infiltration, transient expression.

INTRODUCTION

In vitro genetic manipulations of grain legumes are less amenable compared to most other dicotyledonous crop species, particularly the members of the *Solanacea*e (De Kathen and Jacobsen, 1995). Transformation of some leguminaceous species, particularly large-seeded grain legumes, has so far been difficult to achieve (Somers et al., 2003; Popelka 2004). In general the legumes attracted less attention compared to cereals and other crops, except for soybean and to a lesser extent for pea. Soybean is the first among the stably transformed grain legumes (McCabe et al., 1988).

Stably transformed chickpea plants were first demonstrated by Fontana et al. (1993) and *Agrobacterium* mediated gene transfer by co-cultivation technique was carried out in their study. The presence of integrated DNA was also proved by southern hybridization. *Agrobacterium*-mediated genetic transformation of chickpea was also reported by the other researchers (Kar et al., 1997; Krishnamurthy et al., 2000; Polowick et al., 2004; Senthil et. al., 2004; Tewari, 2004; Sarmah et al., 2004; Sanyal et al., 2005).

Although chickpea is habitually susceptible to *Agrobacterium* infection, the transformation is quite difficult and the rates of transformation frequency is very low. The limiting factors in the production of transgenic plants has been the lack of effective means to introduce foreign genes into elite germplasm. However, the development of novel transformation techniques, by-passing limitations compelled by cell culture constraints, has allowed the transformation of almost all major crops including legumes and woody species. In that context, *In planta* transformation techniques has been developed for various legume species (either with *Agrobacterium* or with electroporation of intact meristems) (Feldman and Marks., 1987, Chowrira et al., 1995). Since the time consuming and laborious tissue culture stages are bypassed, *In planta* transformation methods are advantageous over

*Corresponding author. E-mail: akbulut@erciyes.edu.tr.

conventional transformation methods. However, utilizing the intact and differentiated plant tissue in some of these techniques decreases the transformation efficiency, and obtaining the chimeric plants is very likely.

To our knowledge, *in planta* transformation techniques have not been studied in chickpea transformation. In this study, we aim to investigate the efficiency of an *in planta* transformation technique on chickpea seedlings.

MATERIALS AND METHODS

Plant material

Five Turkish chickpea cultivars (Gökçe, Er, Akçin, Uzunlu and Küsmen) were used throughout the *Agrobacterium* mediated transformation studies. The Cultivars were kindly donated by Dr. Ismail Küsmenoglu and Field Crops Research Institute of Turkish Ministry of Agriculture and Rural Affairs.

Bacterial strains and plasmids

Three *Agrobacterium tumefaciens* strains, C58C1 (Deblaere et al., 1985), EHA105 (Hood et al., 1993) and KYRT1 (Torisky et al., 1997) were used throughout the *Agrobacterium* mediated transformation studies. YEB (Yeast Extract Broth), supplemented with necessary antibiotics was used for the growth of *Agrobacterium* cultures

The Binary vector pTJK136 (Kapila et al., 1997), which is a derivative of the pTHW136 contains a gene coding for streptomycine/spectinomycine adenyl transferase gene as bacterial selection marker and an intron containing *uidA* (GUS) gene, and sequences of neomycine phosphotransferase-II (*npt-II*), conferring resistance to kanamycin between the border sequences, was used.

Agrobacterium induction medium

Induction medium was composed of 4.3 g/L MS salts (M5524, sigma), 20 mM MES (2-[N-morpholino]ethanesulfonic acid), 200 µM acetosyringone (3',5'-dimethoxy-4-hydroxyacetophenone) and 0.5 % glucose. 2 g tobacco leaf extract in 2 ml of induction medium was also added to 100 ml induction medium.

Surface sterilization and seed germination

Chickpea seeds were surface sterilized in 2.5% sodium hypochlorite solution containing 5 - 6 drops of Tween-20 for 30 min. The seeds were washed in sterile distilled water for 3 - 4 times.

Surface sterilized seeds were imbibed in sterile distilled water overnight. The next day, the remaining water was discarded and the seeds were blot dried on a sterile filter paper. 10 seeds were placed on each plate containing semisolid MS basal medium and incubated at 23°C.

Transformation

Agrobacterium strains C58C1/pJTK136, KYRT1/pJTK136 and EHA105/pJTK136 were grown overnight by shaking at 200 rpm at 28°C in YEB medium containing the appropriate antibiotics until OD_{600} reached to 0.8 and centrifuged at 6000g for 10 min (Sigma 3K30 centrifuge). The bacterial cells were later re-suspended in induction medium (pH 5.6, 2X volume of YEB medium) and grown for 18 h by shaking at 150 rpm at 27°C.

The seed coat and one of the cotyledons were removed and the embryo axes (shoot tip and cotyledonary node region) were pricked with a very fine sterile needle and seedlings were dumped into suspension of *Agrobacterium* induction medium. Co-cultivation was carried out by gentle agitation at 100 rpm at 27°C for 2, 8, 16 or 24 h. The seedlings were blot dried on a sterile filter paper and transferred to semisolid MS basal medium and further co-cultivated for 4 days at 23°C. After that 20 explants from each treatment were randomly sampled and the transient gene expression assay was carried out through histochemical GUS staining. Transformation studies were repeated in 3 independent experiments.

Agroinfiltration of chickpea seedlings

Agrobacterium and seedling preparation was the same as the previous experiment. Following the 18 h induction, the bacterial culture was centrifuged and the pellet was re-suspended in induction medium. The final OD_{600} of suspension was adjusted to 2.4. 12 ml of *Agrobacterium* suspension was placed into 40 ml sterile beaker and one cotyledon embryos were dumped into the beaker. The beakers were covered with parafilm and pricked with a needle and placed into infiltration device. The negative pressure was provided from vacuum pump. The various infiltration period and evacuation pressures were applied. A control group, which was not infiltrated and only co-cultivated with *Agrobacterium* suspension, was also included.

GUS histochemical assay

GUS histochemical assay was carried out to assess transient expression of transferred genes in transformed seedlings and tissues (Jefferson, 1987). The shoot and CN region were removed with a scalpel, washed with distilled water and blot dried. The explants were placed in 1.0 mM solution of X-Gluc and incubated overnight at 37ºC. The explants were later transferred to 70% ethanol for de-colorization and then 100% ethanol for dehydration. The explants were examined under stereomicroscope to scan for the GUS expression foci and photographed.

Statistical analysis

Least significance difference test (LSD) which is one of the Post Hoc multiple comparisons of one-way ANOVA of SPSS 10 (Statistical Package for Social Sciences, SPSS Inc., Illinois) was used to detect mean differences in GUS expressing foci on explants subjected to various treatments

RESULTS AND DISCUSSION

It is well known that the transfer of T-DNA was influenced by several factors, including *Agrobacterium* strain and plasmid vector combination, plant genotype, type of explant, condition of inocula and coculture period. Thus, all of the mentioned factors need to be optimized for every transformation

Agro-infection of germinating chickpea seedlings is one of the non-tissue culture based trans-formation strategy that was not previously applied to chickpea transformation. Since there was no previous data concerning chickpea transformation by this technique, impact of various parameters on transient expression, such as *Agrobacterium* strain, co-cultivation

Table 1. Number of GUS expressing foci using different *Agrobacterium* strains and developmental stage.

Strain	Co-cultivation period (h)			
	16 h imbibition		40 h germination	
	2 h	8 h	2 h	8 h
C58C1	95±8.3 a a[a]	130±11.2 a b	78±10.6 a b	79±5.3 a c
KYRT1	98±8.0 a a	133±9.6 a b	92±6.7 b a	128±9.8 b b
EHA105	28±5.0 b a	34±4.4 b b	22 ±2.7 c a	25±3.7 c c

[a]If different, the first and second letters indicates statistical difference (p<0.05) in columns and rows for *Agrobacterium* strain and developmental stage at different co-culture period, respectively.

period in liquid induction medium, developmental stage, wounding, infiltration and genotype effect, was investigated. Furthermore, for each parameter, viability percentages of the seedlings were also determined.

Agrobacterium strains

In this study, transformation efficiency of three different strains of *A. tumefaciens* (C58C1, KYRT1, and EHA105) was determined. None of the explants (wounded or non-wounded) that were not treated with *Agrobacterium* exhibited positive GUS results.

All (100%) of the chickpea seedlings (with one cotyledon) showed transient GUS expression when treated with the C58C1 and KYRT1 strains of *Agrobacterium*. The EHA105 strain was not as efficient as C58C1 and KYRT1 strains. Only 85% of chickpea seedling showed GUS expression when infected with the EHA105 strain. With respect to GUS expressing foci, the C58C1 and KYRT1 strains exhibited better performance when compared to the EHA 105 strain. The cumulative results are given in Table 1. Statistical analysis revealed the KYRT1 strain as the most efficient strain. On the other hand, the EHA105 was found to be the least efficient strain in this experimental system.

The influence of three *Agrobacterium* strains Octopine LBA 4404, Nopaline C58C1, Succinamopine and EHA 105 was also evaluated for pea (*Pisum sativum*) transformation. Transformation efficiency was 8.2, 2.2 and 1.0 % for EHA105, C58C1 and LBA4404, respectively (Orczyc and Orzyc, 2000).

Superiority of KYRT1 strain over nopaline and succinamopine-type *A. tumefaciens* strains were demonstrated previously on soybean cotyledonary nodes and compared to EHA105, this strain exhibited 2.5 to 3 fold higher GUS expression (Torisky et al. 1997). Since it was originally isolated from soybean, which also belongs to Leguminaceae, better performance of KYRT1 strain on chickpea was also expected . Furthermore, compared to C58C1 and EHA105, KYRT1 also yielded higher transformation frequencies in lentil (Çelikkol, 2002). Likewise, Husnain et al. (1997) reported a higher marker gene

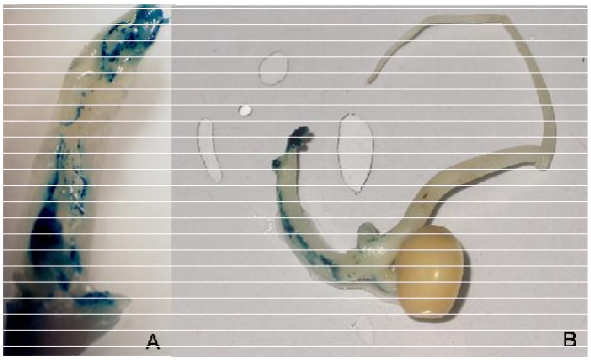

Figure 1. Histochemical GUS staining of explants obtained from seedlings wounded and incubated with induced KYRT1 strain of *Agrobacterium* for16 h and cocultivated for 4 days. **A**. Root and cotyledonary nodes of the embryo were removed after transformation. **B**. intact one cotyledon seedling after transformation.

expression by using the supervirulant A281 strain of *A. tumefacens* in embryo explants of chickpea when compared to C58C1. Among the tested *A. tumefaciens* strains, KYRT1 appeared to be the best in our experimental system and it was utilized in further experiments. A representative experimental result is given in Figure 1.

Micro-wounding

It is well known that several diffusible chemicals are released from wounded tissues such as phenolics and polysaccharides which are potent activating factors of the Agrobacterium virulence (Stachel et al., 1985)

In our experiments a considerably higher transient GUS expression was observed in wounded tissues when compared to non-wounded explants (Figure 2). In 2 and 8 h of co-cultivation duration, 30 and 37% difference were obtained between needle injured and non-injured explants, respectively. Mean GUS foci difference between wounded and non-wounded seedlings was found to be statistically significant (p<0.01).

A number of literature data also demonstrated an enhanced transformation frequency upon wounding. Pre-wounding of sunflower shoot apices or banana meriste-

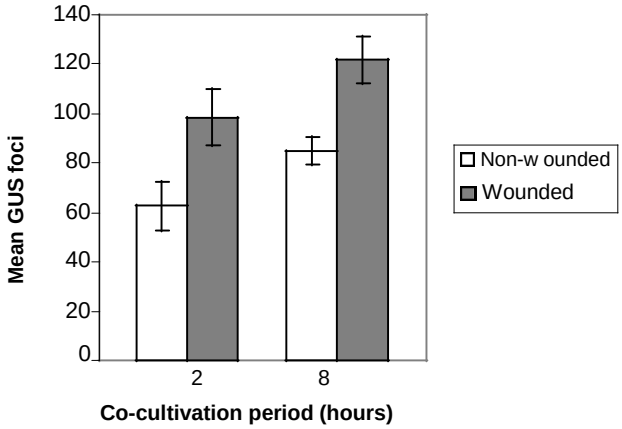

Figure 2. Effect of wounding on transient GUS activity of seedlings (with one cotyledon) wounded or non-wounded seedlings were incubated for different durations (2 and 8 h) in *Agrobacterium*, co-cultivated for 4 days and stained for GUS activity.

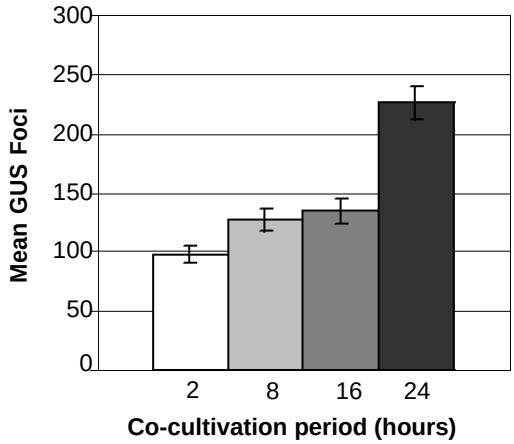

Figure 3. Effect of *Agrobacterium* co-cultivation duration on the number of GUS expressing foci on wounded seedlings.

matic tissues with microprojectile or glass beads has proven to enhance the transformation efficiencies (Grayburn and Wick, 1995). More recently, gene gun mediated micro-wounding was shown to enhance *Agrobacterium*-mediated transformation in sunflower (Lucas et al., 2000), carnation (Zuker et al., 1999) and soybean embryogenic clumps (Droste et al., 2000). Similarly, lentil cotyledonary nodes wounded with a needle or particle bombardment yielded higher transient GUS expression (Çelikkol, 2002). Sonication assisted micro-wounding was also found to enhance the efficiency of *Agrobacterium* mediated transformation by introducing large number of micro-wounds into the target plant tissue (Trick and Finer, 1997; Santarem et al., 1998).

Infection period (co-cultivation in liquid induction medium)

Effect of seedling co-cultivation duration in liquid induction medium was another parameter that was investigated in this study. Four different time intervals (2, 8, 16, 24 h) were used to test the optimal co-cultivation period of wounded seedlings with KYRT1 strain. Extending the co-cultivation period remarkably increased the number of GUS expressing foci (Figure 3). The mean numbers of GUS expressing foci were 98, 127, 134 and 226 for 2, 8, 16, and 24 h co-cultivation duration, respectively. There were more than 2 fold differences between 2 and 24 h of incubation period in terms of GUS expressing foci.

The mean GUS foci difference between 2 and 24 h of co-cultivation period was found to be statistically significant (p<0.01). The prolonged co-cultivation period might cause an increase in the number of the induced bacteria attaching the plant tissue (Dong and McHughen, 1991; Dong et al., 1991; De Bondt et al., 1994; Cervera et al., 1998; Sakae et al., 2001). Consequently, transformation efficiency also increases although bacterial overgrowth decreases the viability of the seedlings.

The effect of the co-cultivation period on *Agrobacterium* mediated transformation has also been reported in a number of plant species (Holford et al., 1992; Muthukumar et al., 1996; Mohan and Krishnamurthy, 2003). Warkentin (1992) reported that a longer co-culture period in lentil transformation resulted in a greater GUS expression. Rohini and Sankara (2000a) demonstrated the effect of co-cultivation period on transient GUS expression percentage of peanut embryos. They also reported that 16 and 24 h of cocultivation period were better than 2 and 4 h. The same group also applied a longer (24 h) incubation time in transformation experiments conducted on safflower (*Carthamus tinctorius*) (Rohini and Sankara, 2000b). Similar to our results, in these studies co-cultivation of explants for 24 h or more generally provided the best transformation frequency. Also, prolonged co-cultivation periods of 6 - 7 days increased transformation efficiency in flax (Dong and McHughen1991).

Effect of vacuum infiltration

The vacuum infiltration is one of the *Agrobacterium* based transformation systems that is considered useful for enhancing the transformation efficiency (Bechtold and Ellis, 1993; Tjokrokusumo et al., 2000; Qing et al., 2000; Trieu et al., 2000; Mahmoudian et al., 2002). We tried various durations and evacuation pressures to enhance the efficiency of the transformation system. To our knowledge, effect of vacuum infiltration on the efficiency of *Agrobacterium* mediated transformation systems has not been investigated in chickpea yet. Three different evacuation pressures (200, 400, and 600 mmHg) and two different evacuation time intervals (20 and 40 min) were tested. As a control non-evacuated conventional *Agro-*

Table 2. Effect of infiltration pressure (0, 200, 400 and 600 mmHg) and durations (20 and 40 min) on transient GUS expression in seedlings with one cotyledon.

Evacuation Pressure (mmHg)	Evacuation period (min)			
	20 min		40 min	
	GUS Foci	Viability (%)	GUS Foci	Viability (%)
0	31±7 a	80	48±8 a	82
200	49±11 b	87	51±6 a	84
400	53±14 b	80	43±5 a	78
600	13±4 c	50	14±3 b	10

Different letters indicates statistical difference (p<0.05) in columns.

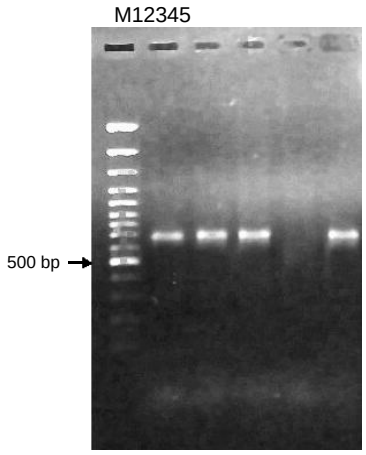

Figure 4. PCR amplification of *npt*II genes. M, DNA ladder, 1, 2 and 3 putative transgenic plants, 4 non-transgenic control, and 5 amplification from plasmid DNA (pTJK136).

bacterium transformation was also included. An enhanced transient GUS expression was observed in explants subjected to infiltration for 20 min. Prolonged duration of infiltration time did not enhance transient GUS expression (Table 2).

The mean difference of non-infiltrated and infiltrated groups was statistically significant (p<0.05) at 20 min evacuation period. For both 20 and 40 min infiltration duration, 600 mmHg evacuation pressures were found to be statistically different from other groups. Since 600 mmHg evacuation pressures were damaging the seedlings, GUS expressing seedling percentage was very low. Similar observations were also recorded in transformation experiments conducted on lentil cotyledonary nodes, where a decrease was observed in transient GUS activity at higher evacuation pressure (Mahmoudian et al., 2002)

Genotype effect

Agrobacterium strains and interaction of *Agrobacterium* strains and plant genotypes is critical for successful transformation of plants. Optimum transformation conditions (KYRT1, 16 h germinated seedlings, and 24 h infection time) were utilized to evaluate the influence of genotype on the transformation system. Average number of 116, 135, 162, 180, and 190 GUS expressing foci was counted on, Akçin, Er, Küsmen, Gökçe and Uzunlu cultivars, respectively. Statistical analysis revealed that Uzunlu and Gökçe were the best responding genotype under these experimental conditions. Presence of GUS activity was also analyzed in one month old transformants of Akçin and Gökçe cultivar. 30 - 40% of one month old transformed plants exhibited GUS histochemical activity in a chimeric manner. In those plants presence of the genes were also confirmed by PCR amplification with *npt-II* specific primers (Figure 4).

The genotype effect in *Agrobacterium*-chickpea interactions were also demonstrated for oncogenic (Islam et al., 1994; Karakaya and Özcan, 2000) and non-oncogenic (Krishnamurthy et al., 2000) strains of *Agrobacterium*. In terms of transformation efficiency, Krishnamurthy et al. (2000) found the Turkish cultivar (Turkey) to be the most efficient among three other chick-pea cultivars (PG1, Chafa and PG12).

Agrobacterium infection of germinating seeds of chick-pea devoid of one cotyledon was investigated for the first time in the course of this study.

Conclusion

Plant genotype, bacterial strains, *Agrobacterium* infection methods (vacuum infiltration versus conventional infection), and co-cultivation period significantly affected the transient GUS activity. Transient GUS activity was not significantly affected from developmental stages. Prolonged co-cultivation period enhanced the gus activity more than 2 times in chickpea seedlings. Since there was no significant difference between infiltrated and non-infiltrated groups at 40 min of infection period, the effect of prolonged co-cultivation time was better when compared to vacuum infiltration.

ACKNOWLEDGEMENTS

This project was supported by the following grants: BAP-2005 (R)-08-11-03, BAP-2002-07-02-00-14.

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