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Comparison of transformation efficiency of five *Agrobacterium tumefaciens* strains in *Nicotiana Tabacum* L.

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Abstract

Agrobacterium mediated transformation has been widely used for research in plant molecular biology and for genetic improvement of crops exploiting its tremendous ability to transfer foreign DNA to plants. In this study, the transformation efficiency of five *Agrobacterium tumefaciens* strains GV2260, LBA4404, AGL1, EHA105, and C58C1 was evaluated in *Nicotiana tabacum* L. cultivar Samsun. The Agrobacterium strains contained the recombinant binary vector pBin19 harboring beta-glucuronidase uidA gene under 35S promoter. Neomycin phosphotransferase (nptII) gene was used as a selectable marker at a concentration of 100 mg L-1 kanamycin. The expression of uidA gene in regenerated T0 plants was firstly analyzed by GUS histochemical analyses and later on confirmation of presence of the nptII and uidA genes in regenerated plants was determined by PCR. The highest transformation rate (20%) was obtained with the Agrobacterium strain LBA4404, followed by EHA105, GV2260, C58C1 and AGL1. The higher transformation efficiency achieved in our studies make LBA4404 Agrobacterium strain optimal for functional genomics and biotechnological applications in tobacco plants.

Key words: Genetic transfer, Comparison, Efficient agro strains, Plant transformation

Introduction

Plant transformation employs the use of *Agrobacterium tumefaciens* in most of the cases which is a naturally occurring soil borne pathogenic bacterium that causes crown gall disease. Naturally *Agrobacterium tumefaciens* infects the wound sites in dicotyledonous plant resulting in the formation of the crown gall tumors. In 1907, this bacterium was reported as the causative agent for the crown gall was for the first time (Smith and Townsend, 1907). The crown gall disease is due to the transfer of a specific fragment "T-DNA" (transfer DNA) from a large tumor-inducing (Ti) plasmid within the bacterium to the plant cell (Zaenen et al., 1974).

T-DNA is integrated into the plant genome and ultimately leading to the development of the crown gall phenotype (Chilton et al., 1977). Two bacterial genetic elements are required for T-DNA transfer to plants. The first element is the T-DNA border sequences that consist of 25 bp direct repeats flanking and defining the T-DNA. The borders are the only 12 sequences required in *cis* for T-DNA transfer (Zambryski et al., 1983). The second element consists of the virulence (*vir*) genes encoded by the Ti plasmid in a region outside of the T-DNA. The *vir* genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome (Godelieve et al., 1998).

The transgenic tobacco plant expressing foreign genes was reported at the beginning of the last decade for the first time, although many of the molecular characteristics of this process were not discovered at that moment (Herrera-Estrella, 1983). With the passage of the time, as plant sciences developed, a great progress in understanding the *Agrobacterium*-mediated gene transfer to plant cells has been explored. The significant advantages *of Agrobacterium*-mediated transformation have been reported over direct transformation methods. The reduced copy number of the transgene has been notably observed resulting in fewer problems with transgene cosuppresion and instability (Koncz et al., 1994, Hansen et al., 1997). *Agrobacterium*-mediated transformation additionally is a single-cell transformation system and not forming mosaic

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plants, which are more frequent when direct transformation, is used (Enriquez-Obregon et al. 1997, 1998). Many researchers have published reviews on plant transformation using *Agrobacterium tumefaciens* and the molecular mechanisms involved in this process during the last few years (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). Tobacco is an agricultural product processed from the leaves of plants in the genus *Nicotiana*. It can be consumed, used as a pesticide and, in the form of nicotine tartrate, used in some medicines. It is most commonly used as a drug and is a valuable cash crop for countries such as Cuba, India, United States, China and many other countries. The chief commercial species, *N. tabacum*, is believed native to tropical America, like most Nicotiana plants, but has been so long cultivated that it is no longer known in the wild. *N. rustica*, a mild-flavored, fast-burning species, was the tobacco originally raised in Virginia, but it is now grown chiefly in Turkey, India, and Russia (Villegier et al. 2003). z

The present studies was conducted to evaluate the transformation efficiency of five *Agrobacterium tumefaciens* strains, GV2260, LBA4404, AGL1, EHA105, C58C1, harboring the plasmid pBin19 containing beta-glucuronidase *uidA* gene under 35S CaMV promoter (Figure 1). The expression of *uidA* gene in regenerated T_0 plants was firstly analyzed by GUS histochemical analyses and later on confirmation of presence of the *nptII* and *uidA* genes in regenerated was determined by PCR.

Materials and Methods

Electroporation of plasmid pBin19 (p35S GUS-INT) in competent cells of Agrobacteria (LBA4404, GV2660, C58C1, EHA105 and AGL1) was done using Bio-Rad electroporation device (# 165-2105). Competent cells of each bacterium (100µL) were mixed with 2µL of plasmid (100ng) and placed on ice. The electroporation device was set at a capacitance of 25µF, voltage 2.2kV with a 200 ohms resistance. After electroporation, cells were resuspended in 1mL of SOC medium and incubated at 28ºC for 2-3 hours at 200 rpm. Culture was spread on plates containing LB medium and 50 mg/L of kanamycin for selection of transformed cells. The plates were incubated at 28ºC for 24-48 hours. Transformed Agrobacterium cells were selected in the form of distinct colonies. Positive clones were screened out by PCR using gene specific primer to amplify *uidA* and *nptII* genes. PCR positive colonies were inoculated into 10 ml of YEP medium supplemented with 50mg/L rifampicin and 100 mg/L kanamycin, and further incubated at 28^{o}C for 48 hours shaking at 200 rpm.

The five different *Agrobacterium* strains (LBA4404, GV2660, C58C1, EHA105 and AGL1) each harboring the same binary vector were used to transform *Nicotiana tabacum* L. cultivar Samsun using a standard protocol (Horsch et al. 1985). Tobacco seeds were surface sterilized for 30 minutes with 30% (v/v) sodium hypochlorite solution. Sterilized seeds were rinsed 5-7 times with sterile water, after drying; they were subsequently spread onto petri dishes containing solid ½ MS medium. Young leaves discs from tobacco plants growing in vitro were used. Tobacco leaf transformation and regeneration were performed as described by Albani et al. (1992) and Horsch et al. (1985). Neomycin phosphotransferase (*nptII*) gene was used as selectable marker at a concentration of 100 mg/L kanamycin. All plant tissue cultures were incubated at 24±2ºC under fluorescent light at 100 µmol $m^{-2}s^{-1}$ with a 16/8 hour (light/dark) photoperiod.

Callus formation was observed 3-4 weeks after the experiment. Regenerated shoots were cut from the attachment points to callus and transferred to jars containing MS media to induce root formation and further development. When roots become stronger, regenerated plants were removed from jars, transferred to soil in growth chamber and then 4 weeks later to greenhouse. When flowers developed they were covered with nylon bags to prevent cross pollination.

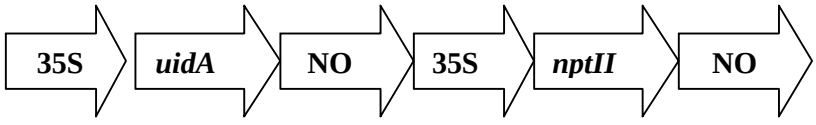

Figure 1. Schematic representation of pBin19 containing beta glucuronidase *(uidA*) and neomycin phosphotransferase (*nptII*) genes driven by cauliflower mosaic virus (CaMV35S) promoter and nopaline synthase (NOS) terminator in between right and left border.

The expression of *uidA* gene was studied through histochemical X-Gluc assay. GUS solution was prepared containing 10mg/L X-gluc, 10mM EDTA, 100mM NaH_2PO_4, 0.1% Triton X-100 and 50% methanol, (pH was adjusted to 8.0). The GUS solution was protected from light. The regenerated shoots transformed by various agrobacteria were dipped in X-Gluc solution in an eppendorf and kept at 37^0C for one hour. The percentage of plants positive for GUS histochemical analysis was recorded.

Genomic DNA was extracted and purified from leaves of regenerated tobacco plants based on the protocol of Li et al. (2001). PCR was carried out using specific primer pairs to amplify *uidA* and *nptII* genes in putative transgenic tobacco plants. PCR was performed in a total reaction mixture volume of 20 µL containing 1X reaction buffer, 50 ng of DNA template, 1.5 mM MgCl2, 1 mM of each dNTPs, 10 ng of each primer and one unit of Taq DNA polymerase. PCR was carried out in a thermal cycler using the following conditions: initial denaturation at 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 58ºC for 40 seconds (64ºC for *uidA* gene), extension at 72ºC for 40 seconds, followed by a final extension at 72ºC for 7 min. The plasmid was used as positive control whereas the DNA isolated from untransformed plants was used as negative control. Amplified DNA fragments were electrophoresed on 1.0% agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) light.

The transformation efficiency in transformants growing on regeneration and rooting selection medium was calculated. Transformation efficiency was determined by dividing the total number of transgenic plants (identified by PCR), by the number of explants inoculated, and then multiplied by 100. A randomized design was applied to the experiments. Three replicates per treatment were used in each experiment. The data were subjected to analysis of variance and analyzed properly.

Results and Discussions

The *Agrobacterium tumefaciens*-mediated transformation method is commonly used to produce transgenic plants as it has several advantages as compared to direct gene transfer methods such as particle bombardment, electroporation, and silicon carbide fibers. The advantages are (1) stable gene expression because of the insertion of the foreign gene into the host plant chromosome; (2) low copy number of the transgene; and (3) large size DNA segments can be transferred. Many crops, flowers and trees have been genetically modified using this method that are important from agronomic and horticultural point of view (Ko and Korban, 2004; Lopez et al. 2004). The various *Agrobacterium* strains have been used to accomplish the tasks. This present study deals with the comparative ability of *Agrobacterium* tumefaciens strains GV2260, LBA4404, C58C1, AGL1 and EHA105 harboring the plasmid pBin19 to transform tobacco. For plasmid confirmation, colony PCR was performed on selected colonies of different *Agrobacterium* strains harboring plasmid of interest. Colony PCR amplified bands of ~1.32 kb and 450 bp for beta-glucuronidase *uidA* and *nptII* genes respectively which indicated the positive clones ready to be transformed in tobacco.

The tobacco leaf discs infected with various Agrobacteria on MS medium supplemented with kanamycin showed callus formation. The callus formation was initiated only from the putative transformed leaf discs and the untransformed leaf discs began to yellow after 3-5 days. Later on, during 5-10 days they became brown and did not initiate callus formation. The transformed leaf discs developed into the callus and then the shoots were emerged with in 18-22 days. Accumulating evidences suggested that in vitro tissue culture and micro propagation are necessary for transformation experiments to remodel the aesthetic and growth characteristics of the plants (Jaime and Teixeira, 2003; Siemens and Schieder, 1996; Tang et al. 1999). The roots continued to grow in the putative transgenic plants on rooting medium. All these kanamycin resistant tobacco plants showed normal phenotype. It has been reported that fine balance of *Agrobacterium* and selective agents might be helpful to raise antibiotics resistant transgenic plants (Silva and Fukai, 2001).

The regenerated shoots from were incubated with X-gluc solution and kept 37ºC overnight. The blue colour indicated the transformation of *uidA* gene into regenerated shoots and leaves as compared with non-transformed shoots where no blue colour was observed (Figure-2). The regenerated shoots transformed by LBA4404 shown best results regarding GUS staining assay followed by GV2260, EHA105, C58C1 and AGL1) as indicated by graphical representation. The transformation efficiencies of all *Agrobacterium* strains were significantly different (P\0.05), except between LBA4404 and GV2260. The putative

transgenic plants were subjected to PCR to confirm the presence of *uidA* and *nptII* genes in the plants. Results showed amplification of ~1.32 kb and 450 bp products for *uidA* and *nptII* genes respectively indicating the proper incorporated foreign genes in tobacco genome (Figure-3&4). The ignorable numbers of escapes were found at this stage suggesting that kanamycin resistance conferred by the *nptII* gene constitutes a good selection system for regeneration of transgenic tobacco plants. However, escapes have been reported in leaf disk transformation experiments of 15 cultivated tomato lines using *Agrobacterium* and kanamycin as a selection system (McCormick et al. 1986). All the putative transgenic plants in soil were subjected to PCR and a maximum number of transformants were found positive by strain LBA4404 followed by GV2260, EHA105, C58C1 and AGL1 (Table 1). Hence it was concluded that *Agrobacterium* tumefaciens strains differ in their ability to transform tobacco plants and LBA4404 remains the best strain for a suitable transformation system in tobacco. However it can also be further investigated how many copies of introduced genes can by inserted in receipt genome by these Agrobacteria.

Table 1. Comparison of different Agrobacterium strains on plant regeneration and transformation of Tobacco. LBA4404 strain showed the best result (20%) as compared to other *Agrobacterium* strains.

Agrobacterium Strain	No. of Explants	No. of Resistant Calli	No. of shoots/explant	Rooted shoots (%)	PCR Positive Plants	Transformation Efficiency (%)
LBA4404	60	55	15	90	12	20.0
GV2260	60	53	12	90	9	15.0
EHA105	60	44	10	80	8	13.3
C58C1	60	44	10	75	7	11.6
AGL1	60	30	3	50	5	8.3

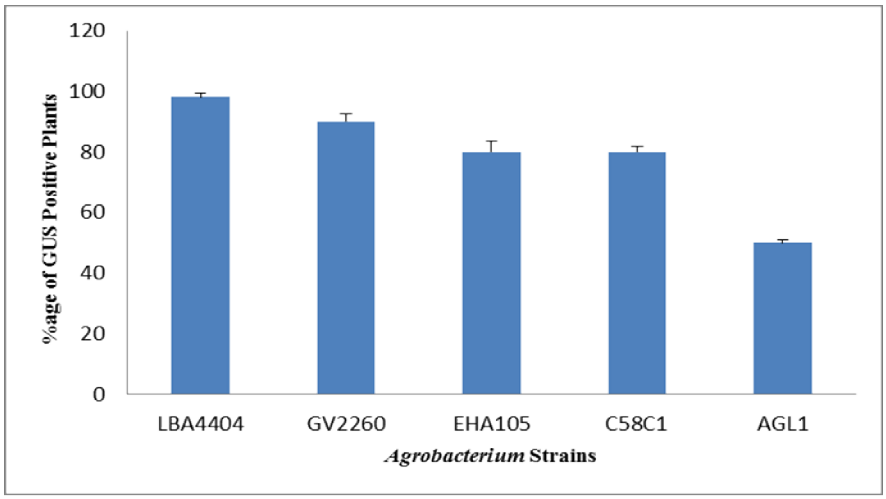

Figure 2. The graph shows the percentage of GUS positive shoots transformed by the different *Agrobacteria* used in the study. Agrobacterium strain LBA4404 resulted in maximum number of transgenic shoots followed by GV2260, EHA105, C58C and AGL1.

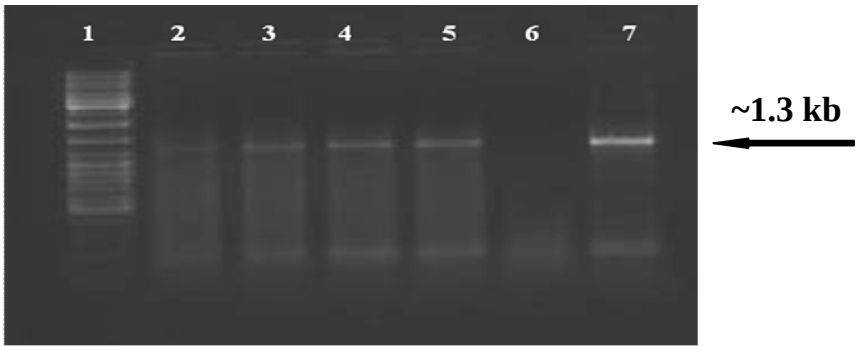

Figure 3. PCR amplification of *uidA* (beta-glucuronidase) gene in transgenic tobacco plants
Lane 1 : DNA Ladder Mix (Fermentas)
Lane 2-5 : Transgenic plants samples (To Progeny) by different agrobacteia
Lane 6 : Negative Control (DNA from non transformed tobacco plant)
Lane 7 : Positive control (plasmid DNA)

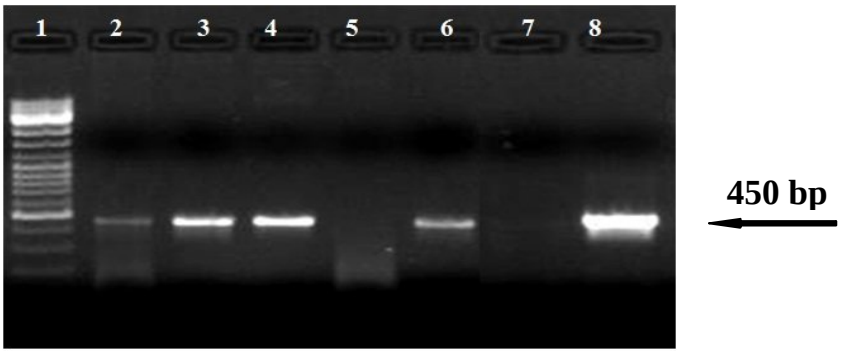

Figure 4. PCR amplification of *nptII* (Neomycin phosphotransferase) gene in transgenic tobacco plants
Lane 1 : DNA Ladder Mix (Fermentas)
Lane 2-6 : Transgenic plants samples (T_o Progeny) by different bacteria
Lane 7 : Negative Control (DNA from non transformed tobacco plant)
Lane 8 : Positive control (plasmid DNA)

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