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Full Length Research Paper

The optimization of voltage parameter for tissue electroporation in somatic embryos of *Astragalus chrysochlorus* (Leguminosae)

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Somatic embryo tissues of *Astragalus chrysochlorus* were transformed with the β-glucuronidase (GUS) and neomycin phosphotransferase II (npt II) genes by electroporation. The effect of electric field strength was tested for transient expression. It was found that 1000 V/cm and 200 μs and 1 pulse was the optimum combination to transform embryo tissues (expression level was 61.5%). Electroporated somatic embryo tissues were positive for GUS expression and PCR analysis for the genes GUS and npt II. After PCR analysis, we found that the efficiency of the somatic embryos with transient GUS expression by electroporation was 48%.

Key words: *Astragalus*, gene transfer, electroporation, β-glucuronidase, neomycin phosphotransferase II.

INTRODUCTION

Electroporation is a simple, efficient method for importing foreign molecules into cells. An electronic impulse makes the cell membrane temporarily permeable to penetrate DNA and other macromolecules, such as RNA, antibodies, peptides or pharmaceuticals into the cell. Electroporation causes minimal biological and toxic side effects and it is a useful technique for cells that show low transfection efficiency when using other methods. To establish a genetic transformation system, it is necessary to have an explant competent for the transformation process and an *in vitro* culture system, which permits a high frequency of regeneration. Ideally, gene transformation systems should be simple, cheap, reproducible and independent of the genotype and inserts the genetic sequence in a stable form (Sharma et al., 2005; Jordan et al., 2008).

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Abbreviations: GUS, β-glucuronidase; **npt II,** neomycin phosphotransferase II; **nos,** nopaline synthetase; **IAA,** indole-3-acetic acid; **MS,** Murashige and Skoog; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **PCR,** polymerase chain reaction.

Astragalus roots from various species have been used in the traditional Chinese medicine, as immunostimulants, hepatoprotectors, antiperspirants, diuretics, etc., and for the treatment of nephritis, diabetes, leukemia and cancer (Tang and Eisenbrand, 1992). In the district of Anatolia, located in South Eastern Turkey, an aqueous extract of the roots of *Astragalus* is traditionally used against leukemia and for its wound-healing properties (Bedir et al., 2001). Thus, development of transformation systems in *Astragalus* should be useful to modify genes to improve the yield of biologically active metabolites. Also, the leguminosae contain many economically important forage crops and there is general interest in developing transformation systems for these species. While a number of species have been regenerated from tissue cultures, only a few legumes have been stably transformed (examples include *Medicago*, *Lotus*, *Trifolium*, *Onobrychis*, Chickpea, Soybean, Pea and Common bean, Lentil and fewer of them was transformed via electroporation) (Somers et al. 2003; Eapen, 2008).

So far, limited number of genetic transformation studies have been reported for *Astragalus* spp. For example, cells, cotyledon segments and seedlings have been transformed via *Agrobacterium* and polyethylene glycol in *Astragalus sinicus* (Xue-Bao et al., 1994; Cho et al., 1998;

Table 1. Primers used in PCR analysis.

Primer	Sequences
GUS Forward Primer	5'-GGTGGGAAAGCGCGTTACAAG-3'
GUS Reverse Primer	5'-GTTTACGCGTTGCTTCCGCCA-3'
npt II Forward Primer	5'-ATCGGGAGCGGCGATACCGTA-3'
npt II Reverse Primer	5'-GAGGCTATTCGGCTATGACTG-3'

Cho and Widholm, 2002) and also hypocotyl segments of *Astragalus melilotoides* plantlets were inoculated with *Agrobacterium* (Zhang et al., 2008).

The main objective of this study is to develop an efficient method for *Astragalus* genetic transformation by electroporation. For this purpose, a transient expression study was performed by using somatic embryos as an explant source. Electroporated tissues analysed by GUS assay and the detection of the genes GUS and nptII were done by using convencional PCR

MATERIALS AND METHODS

Seeds were collected and classified by Prof. Dr. Zeki Aytaç (Gazi University, Faculty of Arts and Science, Department of Biology, Turkey) and a voucher specimen was deposited in the herbarium at Istanbul University, Faculty of Science Herbarium (ISTF).

pBI 121 (Clontech) vector carries the GUS and npt II genes. GUS expression is driven by the 35S RNA promoter of Cauliflower mosaic virus (CaMV) and npt II expression is driven by the nopaline synthetase (nos) promoter. Plasmid DNA was extracted by using GeneJET™ Plasmid Miniprep Kit (K 0502, Fermentas). Electroporation assays were carried out based on a protocol developed by Gurel and Gozukirmizi (2000). Torpedo-shaped somatic embryo tissues which were cultured according to Turgut-Kara and Ari (2008) were used as an explant source. Embriyogenic cell suspension cultures were established from 21-day-old calli on Murashige and Skoog (1962) medium supplemented with 0.5 mg/l 2,4-D. Embryogenic calli were transferred to 50 ml Erlenmeyer flasks (0.5 fresh mass) containing 15 ml of MS liquid medium enriched with 0.5 mg/l concentration indole-3-acetic acid (IAA) and agitated on a gyratory shaker (120 rpm, 25 ± 2°C, in dark). After 15 days in suspension, filter-selected torpedo-shaped embryos were selected and transferred to a sterile Petri dish containing 20 ml buffer A (20 m*M* $CaCl_2$, 10 m*M* hepes, 0.4 *M* mannitol, pH 5.6) and incubated for 1 h at room temperature. The embryos were then transferred to a fresh Petri dish with 20 ml buffer A containing 0.3% pectinase and 0.5% cellulase and incubated for 15 min at room temperature. After incubation, 20 embryos were washed twice with sterile water and transferred to each ice-cold 0.4 cm electroporation cuvettes containing buffer A and 50 m*M* KCl, plasmid DNA (30 µg) was added to each cuvette, mixed, incubated for 30 min on ice and electroporated into embryos using a combination of seven voltage parameters (250, 500, 750, 1000, 1250, 1875 and 2500 V/cm) with 10 µFd capacitance value and one pulse upon an electroporation device (Multiporator®, Eppendorf). Two replicates were used for each treatment and the experiment was repeated three times.

GUS expression was assayed histochemically as described by Jefferson (1987). Two days after electroporation, embryos were incubated overnight at 37°C in GUS staining solution containing 1.5 mM potassium ferrocyanide, 1.5 mM potassium ferricyanide, 0.9% Triton X-100, 0.3% 5 bromo-4 chloro-3 indolyl β-D-glucuronic acid (X-gluc) and 20% methanol. To improve the visualization of the color in the plant tissues, they were clarified with 70% ethanol. For selection of transformed embryos in kanamycin containing medium, electroporated embryos were cultured in MS medium supplemented with 0.5 mg/l of 2,4-D for the first 1 week and then transferred to 100 mg/l kanamycin and 0.5 mg/l of 2,4-D containing selective medium. Embryos electroporated in the absence of DNA were also transferred to the selective medium.

For the detection of GUS and npt II genes, cultured embryos in MS medium supplemented with 0.5 mg/l of 2,4-D and containing 100 mg/l kanamycin were analysed. Genomic DNA was extracted from embryos using GenElute™ Plant Genomic DNA Kit (G2N70, Sigma) and GUS and npt II genes were amplified using PCR (primers shown in Table 1). Reactions were carried out with the following parameters: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final elongation step at 72°C for 10 min.

RESULTS AND DISCUSSION

Transient reporter gene expression is dependent upon biological and physical parameters of transformed tissue. The explant source and energy input are the most important ones (Sharma et al., 2005; Jordan et al., 2008). Thus, to develop efficient electroporation conditions for *Astragalus chrysochlorus*, we investigated the effect of energy input on the transformation. Between the intact tissues, torpedo- and cotyledonary-shaped somatic embryos are the most regenerable ones (Anbazhagan and Ganapathi, 1999). Thus, as an explant source, we used torpedo-shaped somatic embryos. Similarly, Fernandes Da-Silva and Menéndez-Yuffá (2003), who were electroporated different somatic embryos of coffee, found the highest transient GUS gene expression at the torpedo-shaped stage.

In this work, histochemical assays detected GUS expression on the surface of embryos 2 days after electroporation at several voltages. GUS expression was observed with electric field intensities 250 V/cm between 1875 V/cm, but not at 2500 V/cm. Specifically, the 1000 V/cm and 200 µs and 1 pulse condition resulted in the highest percentage of embryos (61.5%) exhibiting cell clusters that express GUS activity (Figure 1), whereas the lowest efficiency of GUS expression (8.33%) was observed at the 250 v/cm and 200 µs and 1 pulse condition (Figure 2). GUS expression was detected on the surface of the somatic embryos in torpedo stage. In fact, when intact cells are electroporated, the level of gene expression is lower when compared with protoplasts (Quecini and Vieira, 2001). This is likely due to the presence of the cell

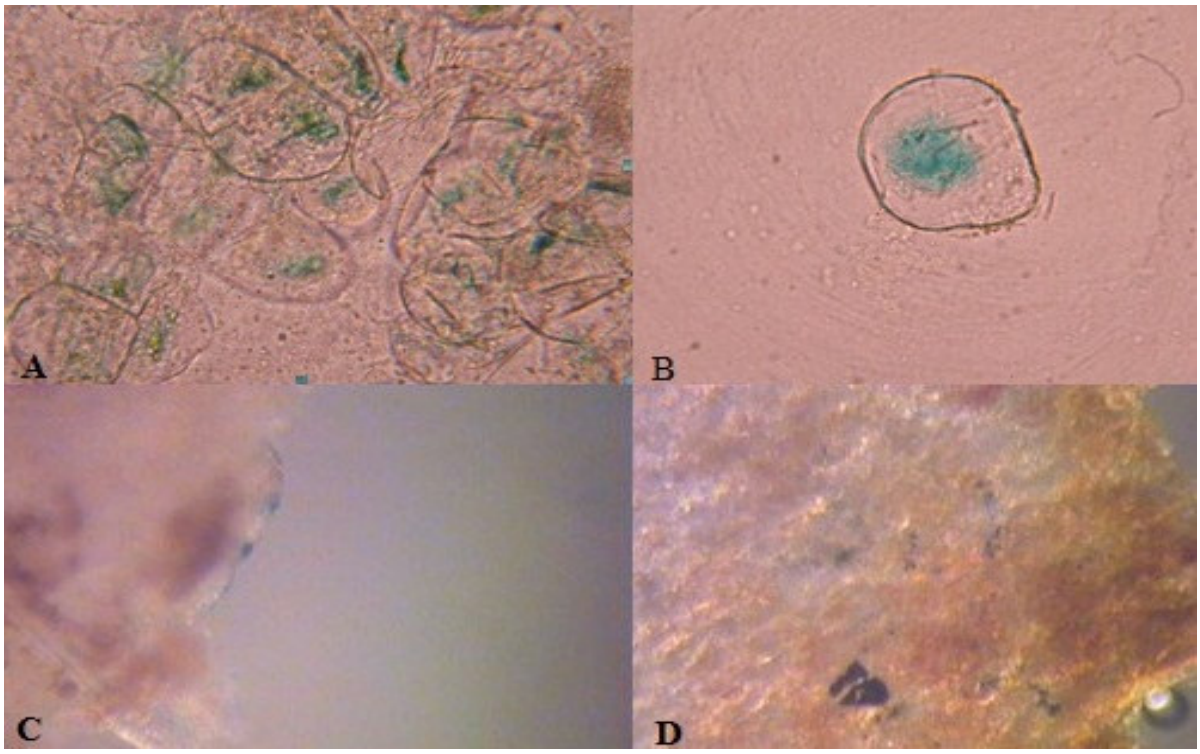

Figure 1. GUS experession at 1000 V/cm and 200 µs and 1 pulse condition. A - B: Cells of the embryogenic tissues of *A. chrysochlorus*, C - D: torpedo-shaped somatic embryo tissues of *A. chrysochlorus.*

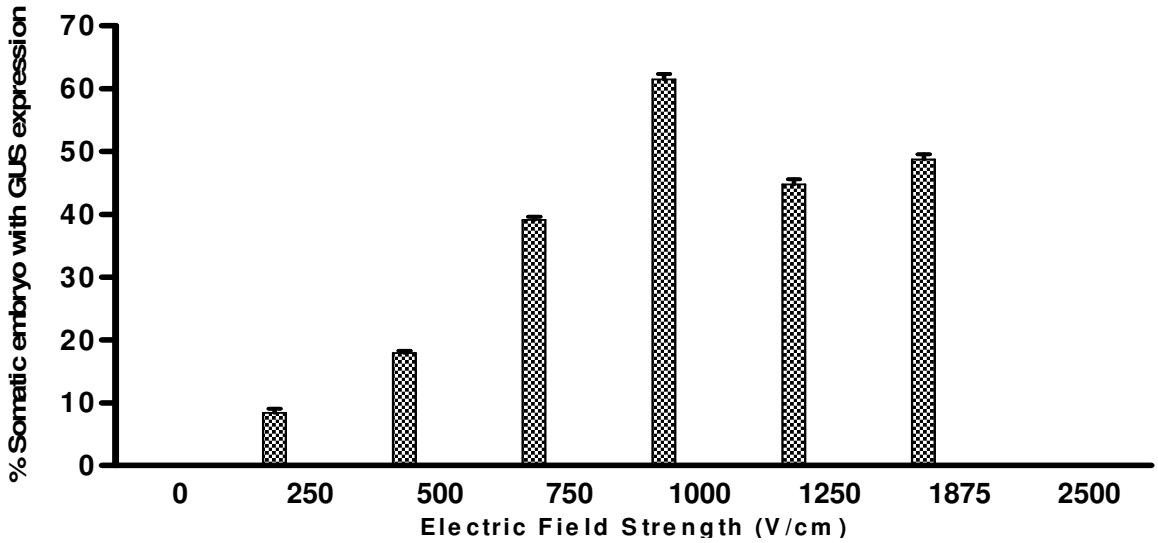

Figure 2. Effect of the 250 - 2500 V/cm electric field strength on GUS expression in somatic embryos of *A. chrysochlorus* (Error bars indicate standard errors).

wall, considered as a barrier that limits the free movement of large molecules such as DNA. In this sense, to improve the DNA incorporation to the cell, one alternative is the application of an enzymatic treatment to remove the pectin present in the cell wall. The pectin inhibits the DNA transference acting as a physical and chemical barrier. To improve the gene expression, electroporated tissues and cells were treated with cell wall digesting enzymes. For this purpose, we use buffer A containing 0.3% pectinase and 0.5% cellulase and incubated tissues for 15 min at room temperature as described in Gurel and Gozukirmizi (2000).

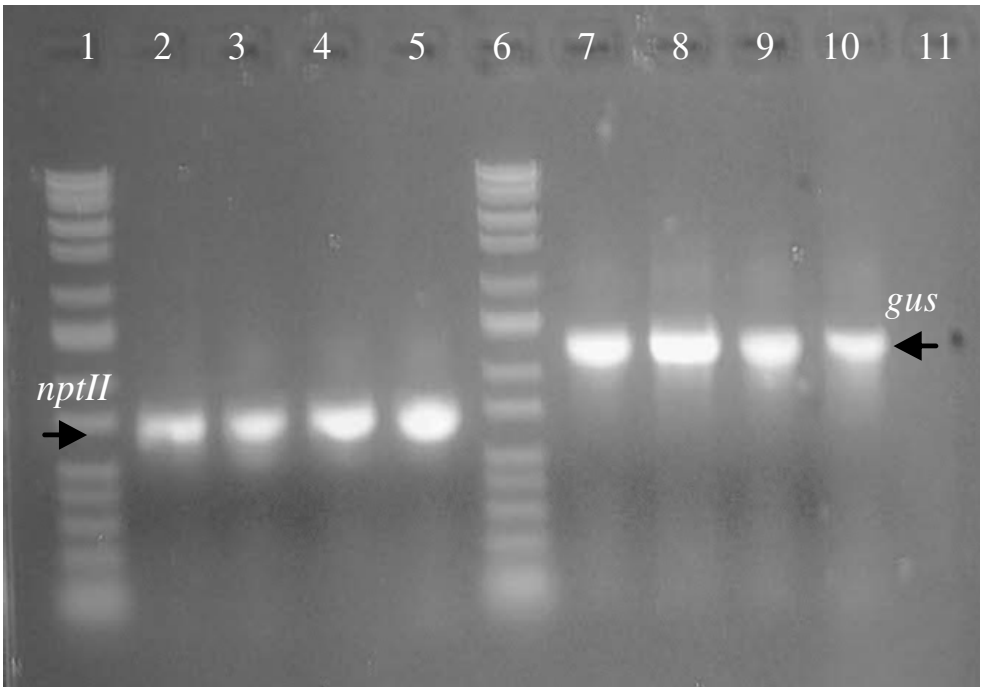

Figure 3. PCR analysis of some transformed embryos which was electroporated on 1000 V/cm electric field strength and grew under kanamycin selection. Lanes 1 and 6: Molecular size marker (Fermentas 1 kb plus), lanes 2 - 5: npt II (0.8 kb), lanes 7 - 10: GUS (1.2 kb) gene positive somatic embryos, lane 11: untransformed somatic embryo as a negative control.

After the transformation procedure, the using of selective agent is critical for eliminating untransformed cells. Here, we examined the effect of the presence of 100 mg/l kanamycin on explants growth. For this purpose, after 1 week from transformation, electroporated embryos were transferred into MS medium containing 0.5 mg/l of 2,4-D and 100 mg/l of kanamycin. Control embryos electroporated without DNA did not grow in the kanamycin selection medium. In a total manner, the viability of the electroporated embryos was about 50% which were alive in 100 mg/l kanamycin at least for four weeks. In 12 embryos which were electroporated, 1000 V/cm and grew in 100 mg/l of kanamycin GUS and npt II genes were detected by PCR (Figure 3). In contrast, 13 embryos that grew in 100 mg/l of kanamycin, neither GUS nor npt II genes were detected by PCR. In this manner, 12 somatic embryos with the presence of GUS and npt II gene indicated as putative transgenic plants. Totally, 45 putative transgenic somatic embryos analysed with PCR, as a result, we found that the efficiency of the somatic embryos of *A. chrysochlorus* with transient GUS expression by electroporation were 48%.

In summary, these results demonstrate that somatic embryos can be used as a target for gene transfer by electroporation of *A. chrysochlorus*. The use of these tissues in transformation experiments is advantageous because we can grow them during all seasons at our tissue culture laboratory and are highly adaptive to live long term in tissue culture conditions.

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