J. Glob. Innov. Agric. Sci., 2022,10(2):101-104.
ISSN (Online): 2788-4546; ISSN (Print):2788-4538
DOI: https://doi.org/10.22194/JGIAS/10.993
http://www.jgiass.com

Co transformation of Gus and npt II genes using Agrobacterium tumefaciens strain GV2260:p35 GUS INT in tobacco variety Samsun Maden 2421

Parisa Pourali Kahriz

Department of Field Crops, Faculty of Agriculture, Ankara University 06110, Dışkapı, Ankara Türkiye.
Corresponding author: pouraliparisa@gmail.com

Co-transformation in Agrobacterium-mediated is known as an important problem. Therefore, this study aimed to co-transform the Samsun Maden variety of tobacco. For this purpose, fresh seeds of cv. Samsun Maden 2421varitey of tobacco was procured from the Bafra county open market in Samsun province, Türkiye. In this study, a well-known transformation through *A. tumefaciens* was used because plants for example tobacco are recalcitrant or difficult to regenerate and fail to respond to other transformation techniques. The result showed successful co-transformation of square-shaped leaf explants on all cultures, excluding 50 mg/l Kanamycin used as a selection pressure. This concentration showed minör escapes to the extent of 1.67%. Indicating that optimizing selection pressure is very important to avoid escapes in co-transformation. Moreover, the regeneration efficiency of explants is reduced considerably with each increase in selection pressure.
Keywords: Agrobacterium tumefaciens, selective genes, reporter genes, transformation, gus expression.

INTRODUCTION

Agrobacterium tumefaciens is a Gram-negative bacterium (Kumar *et al.,* 2004), is used for simple and cheap genetic transformation in plants (Rao and Rohini, 1999), and has the benefit of integration into the genome of the plant (Bourras *et al.,* 2015). T DNA transfer from the Ti plasmid (Bundock *et al.,* 1995). These are easy to maintain and easily synthesized in producing a series of secondary metabolites and exotic proteins that help in inducing transformed plants (Vanisree *et al.,* 2004, Mirjalili *et al.,* 2009). These plants can be used for stable transformation (Zale *et al.,* 2009), functional genomics analysis (Michielse *et al.,*2005), composite plants development (Chabaud *et al.,*2006), studying changing plant metabolic pathways (Xu *et al.,*2021), antibody production (Bishop *et al.,* 1989), the activity of promoter analysis (Langridge *et al.,* 1989), and heterologous protein expression (Marillonnet *et al.,* 2005). Moreover. it is possible to segregate T-DNAs and vector-derived binary vector-derived T-DNA during meiosis from genetically transformed plants in the later progenies (Hatamoto *et al.,* 1990). therefore, transformation through *A. tumefaciens* is a desired technique for plants that are recalcitrant or difficult to regenerate and fail to respond to other transformation techniques. The study aimed to check the co-transformation ability and efficiency of the tobacco cultivar Samsun Maden 2421.

MATERIALS AND METHODS

***Acquisition of the tobacco*:** The fresh seeds of cv. Samsun Maden 2421 was purchased from the open market of Bafra county in Samsun province, Türkiye.
These seeds were sterilized in 50% commercial bleach (2.5% NaOCl) for 20 minutes and rinsed for 3 × 3 min using bidistilled sterilized water. These were germinated on a 1× MS medium to induce plant generation and development.
***Preparation of Explants*:** The leaf disc explants were taken from 28 days grown Samsun Maden 2421 tobacco plants. The seeds were subjected to germination using MS (Murashige and Skoog, 1962) medium containing 30 g/l sucrose (w/v) and solidified with 7 g/l (w/v) agar in sterilized glass Petri dishes at 25°±1°C in a 16-h light photoperiod (Kahriz and Kahriz 2017, 2018). The pH of culture treatments was adjusted to 5.7±0.1 using 0.1 N HCl or 0.1 N NaOH before autoclaving at 105 kPa pressure for 21 min at 121°C.
Agrobacterium tumefaciens strain GV2260 (p35S GUS-INT) was used in the study. It was obtained from the Department of Field crops of Ankara University, Türkiye. It is mentioned that the npt-II selective gene is controlled by NOS promoter and terminator, GUS reporter gene is controlled by 35S promoter and terminator sequences.
The leaf explants of tobacco cv. Samsun Maden 2421 was treated with the suspension of *A. tumefaciens* to check the transfer and integration of nptII (selective gene) and gusA

Kahriz, P.P. 2022. Co transformation of Gus and npt II genes using Agrobacterium tumefaciens strain GV2260::p35 GUS INT in tobacco variety Samsun Maden 2421. Journal of Global Innovations in Agricultural Sciences. 10:101-104.
[Received 17 May 2022; Accepted 20 Jun 2022; Published (online) 24 Jun 2022]
Attribution 4.0 International (CC BY 4.0)

(reporter gene) into the plant genome. *A. tumefaciens*-based selective transformation was tested under selection pressures of 0, 50,100, 150, and _200 mg/l kanamycin + 500 mg/l Duocid Pfizer (Istanbul, Türkiye) for optimization. The Antibiotics were filter-sterilized and added when the autoclaved medium was cooled to about 40°C.

***Inoculation of tobacco leaf explants and induction of shoots*:** *A. tumefaciens* was multiplied in Nutrient broth to OD 600 = 0.24using rifampicin and kanamycin antibiotics (both at 50 $mg \cdot L^{-1}$) before inoculation.

***Inoculation*:** Young tobacco leaves were aseptically cut into about 8×8 mm square disc pieces excluding midribs of leaves and treated with liquid *A. tumefaciens* suspension in a Petri dish for 30 minutes. Inoculated explants were held on sterilized filter papers to avoid accumulated excessive *Agrobacterium*. Thereafter the explants were transferred to MS medium supplemented with 30 g·L-1 sucrose for co-cultivation at 24°C for 48 hours.

Subsequently, these explants were moved to an MS medium containing 500 mg/l Duocid, a broad-spectrum antibiotic with 50, 100, 150, and 200 mg/l Kanamycin, to select the explants. Each Petri dish was sealed with sera brand stretch film and incubated at 24±1°C with a 16 h light photoperiod for shoot induction. The non-inoculated explants were treated as a control treatment. These were treated with water without Kanamycin and Duocid. The Data were collected after 28 days of culture. Each explant was observed to count developing shoots by destructive sampling. Each treatment was compared by taking an average of all parameters within a glass culture bottle exhibited the data for each replicate.

***Histochemical Gus analysis*:** The histochemical test for the presence of the gusA gene was performed following Jefferson *et al.*, (1987) by submerging the leaf explants into GUS staining solution (1mM X-Gluc, 50mM phosphate buffer, 10mM EDTA, and 0.1% Triton x-100) by incubating for 24 h at 38°C. Subsequently, the explants were treated with 96%% ethanol for 72 h by changing ethanol after every 24 h to completely digest the chlorophyll on leaves and noting Gus-based blue-colored expression on leaves.

***Statistical analysis*:** Each treatment contained 60 explants divided equally into four replications of15 explants in each culture bottle. The results were exhibited as the average values ±standard error. The data were subjected to one-way ANOVA. The statistical differences among average values were compared using the LSD test through statistical computer software IBM SPSS 26. (Anonymous 2022).

RESULTS AND DİSCUSSİON

All treated explants showed direct induction of shoots buds on the margins of inoculated leaves after 3-4 days of culture. These shoots buds induced visible shoots after 6-7 days on all explants (Table 1) under all selection pressures.

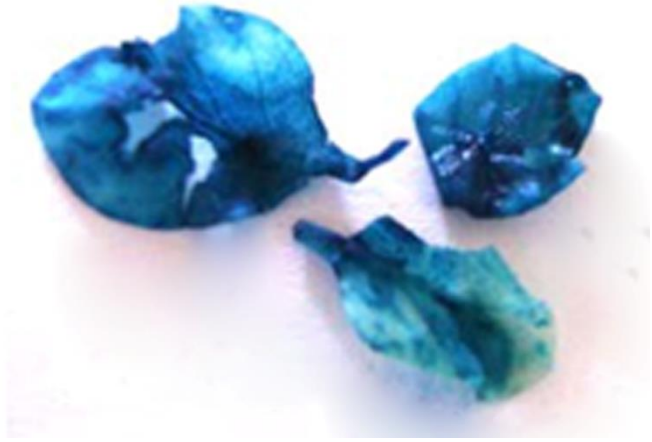

Figure 1. Histochemical GUS expression in plant leaves of Samsun Maden 2421 tobacco variety after treatment with GV2260::p35 GUS INT strain of *A. tumefaciens*.

It was expected that if all developing shoots were co-transformed by *npt II* and *gus uidA* gene, they will grow on a Kanamycin-containing medium and show GUS expression. Non-transformed explants were expected to show albino or development of necrosis on shoots and explants. The results showed 100% shoot regeneration (Figure 1) and gus expression with reduced induction of shoots on explants with each increased concentration of Kanamycin into plants regardless of Kanamycin-based selection pressure. The

Table 1. Shoot regeneration on leaf discs of tobacco Samsun Maden 2421 variety given selection pressure of different Kanamycin concentrations and Gus expression.

Kanamycin concentration (mg/l)	Percentage (%) of regenerated shoots showing tolerance to Kanamycin concentrations	Number of tolerant shoots per explant	Shoot length (cm)	Gus expression percentage (%)
0 (Control)	100.00	23.64a**	10.24a**	0.00c**
50	100.00	18.84b	7.49b	98.33b
100	100.00	16.56c	6.49c	100.00a
150	100.00	9.84d	5.17d	100.00a
200	100.00	3.72e	3.33e	100.00a

**All values shown by different letters in a single column are statistically different at a 0.01 level of significance

results partially confirm the findings of Crane *et al.,* (2006). They reported genetic transformation as a result of co-transformation. There were escapes on 50 mg/l kanamycin selection pressure showing 98.33% Gus positive shoots with escapes. The results are in agreement with Collier *et al.,* (2005). They also confirm that 20- to 60% of induced shoots were induced by the hormones secreted due to the *A. tumefaciens* rather than the integration of genes. Therefore, observance of a careful approach during selection is desired when binary vectors are used to avoid erroneous results. Considering these, the developing shoots on the leaf discs were tested for GUS expression to confirm the co-integration of two genes into the plant genomes. All of the developing shoots except those developing on 50 mg/l Kanamycine showed high levels of expression, showing GUS expression. Samsun Maden 2421 tobacco variety was transformed with *A. tumefaciens* strain GUS INt. Shoot induction occurred at all selection pressures. However, the transformation frequency was reduced at high selection pressure using 200 mg/l kanamycin, confirming Samasun Maden's suitability for genetic co-transformation at appropriate selection pressure (>100 mg/l Kanamycin without escapes.

***Authors' Contributions statement*:** All contributions in terms of idea, implementation of the Project statistical analysis, and writing of the paper was done by the single author Parisa Pourali Kahriz.

***Conflict of interest*:** There is no conflict of interest between the author with any other person.

***Acknowledgment*:** The author thanks the Department of Field Crops, Faculty of Agriculture, Ankara University, Türkiye for support during the study.

REFERENCES

Bishop, A. L., T. J. Burr, V. L. Mittak, and B. H. Katz. 1989. A monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3 and its utilization for indexing grapevine propagation material. Phytopathology.79:993 -998.

Bourras, S., T. Rouxel and M. Meyer. 2015. *Agrobacterium tumefaciens* gene transfer: how a plant pathogen hacks the nuclei of plant and nonplant organisms. Phytopathology.105:1288-1301.

Chabaud, M., A. Boisson-Dernier, J. Zhang, C. G. Taylor, O. Yu, and D. G. Barker. 2006. *Agrobacterium rhizogenes*-mediated root transformation. The Medicago truncatula handbook, version November. pp.1-8.

Collier, R., B. Fuchs, N. Walter, , W. Kevin Lutke, and C. G. Taylor. 2005. *Ex vitro* composite plants: an inexpensive, rapid method for root biology. The Plant Journals.43:449-457.

Crane, C., E. Wright, R. A. Dixon and Z. Y. Wang 2006. Transgenic *Medicago truncatula* plants obtained from *Agrobacterium tumefaciens*-transformed roots and *Agrobacterium rhizogenes*-transformed hairy roots. Planta. 223:1344-1354.

Anonymous.2022. IBM SPSS statistics.pp.26. https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-26.

Hatamoto, H., M. E. Boulter, A. Shirsat, E. J. Croy and J. R. Ellis. 1990. Recovery of morphologically normal transgenic tobacco from hairy roots co-transformed with *Agrobacterium rhizogenes* and a binary vector plasmid. Plant cell reports. 9:88-92.

Jefferson, R. A., T. A. Kavanagh, and M. W. Bevan 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO journal. 6:3901-3907.

Kahriz, M. P., and, P. P. Kahriz. 2018. Improvıng seedlıng growth and tillerıng wıth osmotıc priming treatments ın rice cv. Hamzadere and Osmancık 97. Journal of animal & plant sciences. 28:92-99.

Kahriz, P. P. and M. P. Kahriz. 2017. Somatic embryogenesis on plumule and radicle explants obtained from warm water hydroprimed wheat (*Triticum aestivum* L.) cv. Kunduru and cv. Cakmak Seeds. Sains Malaysiana. 46:35-41.

Kumar, S. V., R. W. Misquitta, V. S., Reddy, B. J. Rao, and M. V. Rajam. 2004. Genetic transformation of the green alga—*Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. Plant science.166:731-738.

Langridge, W. H. R., K. J. Fitzgerald, C. Koncz, J. Schell, and A. A. Szalay. 1989. Dual promoter of *Agrobacterium tumefaciens* mannopine synthase genes is regulated by plant growth hormones. Proceedings of the National academy of sciences. 86:3219-3223.

Marillonnet, S., C. Thoeringer, R. Kandzia, V. Klimyuk and Y. Gleba. 2005. Systemic *Agrobacterium tumefaciens*–mediated transfection of viral replicons for efficient transient expression in plants. Nature biotechnology. 23:718-723.

Michielse, C. B., P. J. Hooykaas, C. A. van den Hondel and A. F. Ram. 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Current genetics. 48:1-17.

Mirjalili, M. H., E. Moyano, M. Bonfill, R. M. Cusido and J. Palazón. 2009. Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules. 14:2373-2393.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum. 15:473-497.

Rao, K. S. and V. K. Rohini. 1999. *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): A Simple Protocol. Annals of botany. 83:347-354.

Vanisree, M., C. Y. Lee, S. F. Lo, S. M. Nalawade, C. Y. Lin and H. S. Tsay. 2004. Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Botanical Bulletin of Academia Sinica. 45:1-22.

Xu, N., Q., X. Yang, M. Wang and M. Guo. 2021. Reconstruction and analysis of a genome-scale metabolic model for *Agrobacterium tumefaciens*. Molecular plant pathology. 22:348-360.

Zale, J. M., S. Agarwal, S. Loar and C. M. Steber. 2009. Evidence for stable transformation of wheat by floral dip in *Agrobacterium tumefaciens*. Plant cell reports. 28:903-913.